The immune response to yellow fever vaccination in aged individuals

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Summary

The immunological competence to fight infections and to generate protective immunity, for example upon vaccination, progressively declines with advancing age. Although the aged immune system has been extensively studied at steady state and in aged animal models, there is only rudimentary understanding on how aging affects the immune response to a primary infection in humans. Involving complex individual systemic immune properties, such investigations have been very challenging particularly with the given restrictions of experimental infections in humans.

In our study, we explored age-related changes in human immunity during experimental, primary immunization with live-attenuated yellow fever (YF) vaccine. In 11 young (median age: 26 years) and 12 elderly (median age: 60 years) vaccinees, we assessed individual viral burden and compared humoral and cellular immunity by advanced flow cytometric analysis over the entire course of the acute infection and up to 3 years after it.

We discovered that aged subjects developed fewer neutralizing antibodies, mounted diminished YF-specific $CD8^+$ T-cell responses and showed quantitatively and qualitatively altered YF-specific $CD4^+$ T-cell immunity. A comparatively late peak in YF viremia suggested impaired infection control and viral clearance in the elderly. Among numerous immune signatures, low *in vivo* numbers of naive $CD4^+$ recent thymic emigrants ($CD4^+$ RTE) prior immunization and peripheral dendritic cells (DCs) in the early phase of the innate response phase were indicative for reduced acute responsiveness and altered long-term persistence of human cellular immunity to YF vaccination in the elderly. Thus, we reveal by this study that essential elements of immune responses such as $CD4^+$ RTEs and DCs affect productive immunity in the elderly, explaining conclusively diminished responsiveness to vaccination with neo-antigens and infection with *de novo* pathogens in aged people.

Zusammenfassung

Mit zunehmendem Alter verringert sich die Fähigkeit des menschlichen Organismus Infektionen erfolgreich zu bekämpfen und, z.B. nach Impfung, einen protektiven Immunschutz aufzubauen. Es wird vermutet, dass die Alterung des Immunsystems eine wichtige Rolle dabei spielt. Wichtige Ergebnisse liefern dazu vor allem tierexperimentelle Studien, welche jedoch die Komplexität menschlicher Immunität, insbesondere in Bezug auf das Altern, nur bedingt abbilden können. Nur ansatzweise erforscht ist der Einfluss immunologischer Alterungsprozesse auf eine primäre Immunantwort im Menschen, was vor allem auf hohe experimentelle und ethische Hürden zurückzuführen ist.

Um ein besseres Verständnis über primäre Immunantworten im Alter zu erlangen, haben wir für diese Arbeit erstmals junge (n=11, Ø=26 Jahre) und ältere (n=12, Ø=60 Jahre) Erwachsene mit einem viralen Erreger experimentell infiziert und die akute Immunreaktion und Entwicklung langlebiger Protektion eingehend untersucht und verglichen. Dafür verwendeten wir den attenuierten Lebendimpfstoff gegen Gelbfieber (GF), der ein hervorragendes Modelsystem darstellt, um vollständige, anti-virale Primärantworten im Menschen zu erforschen.

Wir konnten zeigen, dass ältere Impflinge weniger Gelbfiebervirus-(GFV)-neutralisierende Antikörper produzierten, schwächere GFV-spezifische $CD8^+$ T-Zellantworten erzeugten und sowohl quantitativ als auch qualitativ veränderte GFV-spezifische $CD4^+$ T-Zellantworten generierten. Zudem wiesen ältere Impflinge häufiger eine vergleichsweise späte Virämie im Serum auf, welche durch die altersbedingten Veränderungen der immunologischen Abwehr verursacht sein könnte. Mittels systembiologischer Untersuchung konnten wir weiterhin zeigen, dass vor allem die niedrige Zahl von frisch aus dem Thymus ausgewanderten naiven $CD4^+$ T Zellen, sogenannten $CD4^+$ *Recent thymic emigrants* ($CD4^+$ RTE), sowie der Mangel an dendritischen Zellen vor bzw. am Beginn der Infektion ausschlaggebend für die schlechtere Immunreaktion und niedrigere Langzeit-Immunität bei Älteren war. Aus unseren Untersuchungen schließen wir, dass insbesondere in älteren Menschen die Verfügbarkeit eines breiten Repertoires naiver $CD4^+$ T-Zellen und eine effektive Induktion des angeborenen Immunsystems in der frühen Phase einer primären Infektion kritisch für die erfolgreiche akute Abwehr viraler Erreger und die Ausbildung langlebiger, protektiver Immunität ist.

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1. Introduction

1.1 Pandemic aging of world's population

Aging of mankind has been identified as one of the major challenges of the 21st century. According to the World Health Organization (WHO) numbers of people aged >65 years will rise from 524 million nowadays to 1.5 billion by 2050, which will be 16 % of world's total population (Services, 2011). Accelerated aging is a consequence of increasing life expectancy and declining fertility and will confront societies with enormous socioeconomic challenges especially in the health system. Importantly, rapid aging is not only limited to Western developed nations, but will even more dramatically occur in today's developing countries. For example India's elderly population will almost triple until 2050 from nowadays 60 million to 227 million. The same is true for Brazil, for which an increase of those aged >65 from 20 million in 2010 to 65 million in 2050 is predicted (Gragnolati *et al.*, 2011). As aging is connected with many physiologic alterations, potentially leading to age-related clinical syndromes (Stanziano *et al.*, 2010), it can be considered as a pandemic affecting world's population. Thus, a better understanding of underlying physiological mechanisms of aging is imperative to facilitate efforts for the prevention of disease and the improvement of life quality of elderly people.

1.2 The aging of the immune system

The human immune system, mediating protection against viral, bacterial and parasitic pathogens and malign neoplasms, undergoes a complex transformation with age, eventually leading to a decline in immunological competence. As individuals age, this transformation, also called "immunosenescence", causes an increased susceptibility to infections, resulting in greater morbidity and mortality than in young subjects (Gavazzi and Krause, 2002). For example, in an outbreak of West Nile virus (WNV) infection in New York in 1999, 88 % of the hospitalized patients were at least 50 years old and an age of 75 years or older was the factor most strongly associated with death (Nash *et al.*, 2001). Also the fatal outcomes from influenza virus or respiratory syncytial virus infections were highest in persons aged ≥75 years (Matias *et al.*, 2014). Immunosenescence is furthermore a major contributor to the increasing incidence of malignancies and autoimmunity in the elderly (Goronzy and Weyand, 2012; Fulop, Larbi, Kotb, *et al.*, 2013; Fulop, Larbi, Witkowski, *et al.*, 2013; Goronzy *et al.*, 2013).

Changes associated with immunesenescence are very diverse and a multitude of components of the immune system are affected, as illustrated in fig. 1.1 and outlined in the next chapters. Importantly, though immunological aging appears to be progressive, the decline in immunological function is not linear, but a highly individual process affected by a complex combination of many factors, such as genetic variation (Poland *et al.*, 2014), psychological stress (Bauer, 2008), diet (Maijó *et al.*, 2014), gut microbiota (Rehman, 2012; Duncan and Flint, 2013) and chronic infections (Le Saux *et al.*, 2012). In consequence, longevity and frailty are critically influenced by the individual immunological fitness at advanced age (Ferguson *et al.*, 1995; Remarque and Pawelec, 1998; DelaRosa *et al.*, 2006; Desquilbet *et al.*, 2007; De Fanis *et al.*, 2008; Wang and Casolaro, 2014).

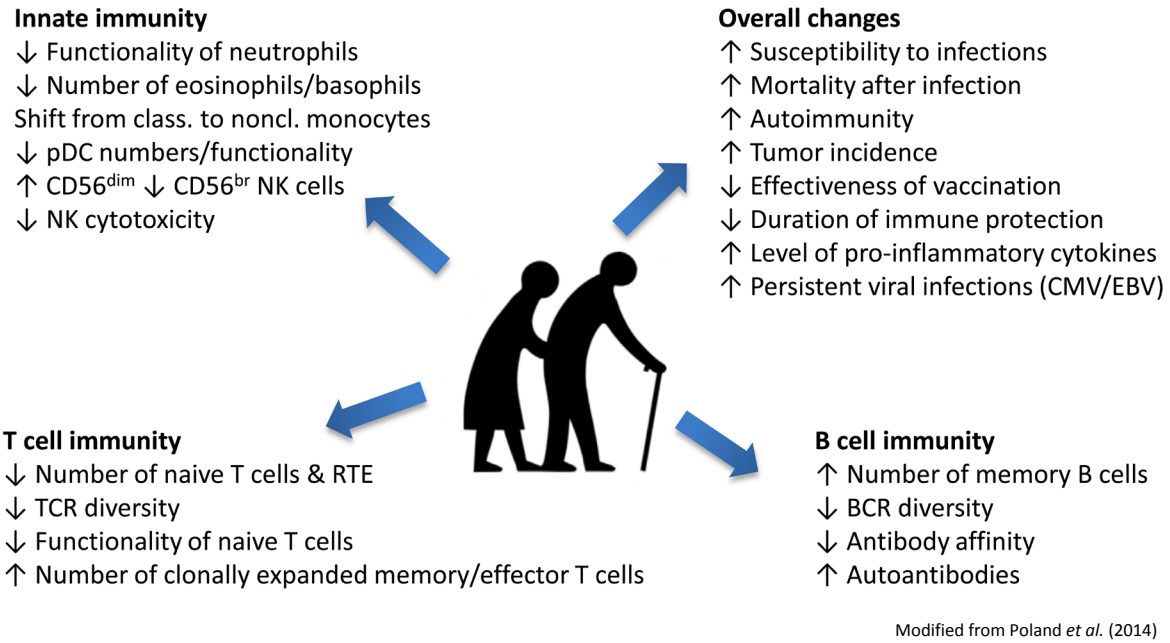


Figure 1.1: Age-associated changes of the human immune system

Much of the current knowledge about the aged immune system at the steady state has been obtained from large population studies (Ferguson *et al.*, 1995; Wikby *et al.*, 2008). Moreover, the impact of chronic infections on the immune system has been extensively studied in young and aged individuals (Brunner *et al.*, 2011; Fülöp *et al.*, 2013; Sansoni *et al.*, 2014). Further research has been conducted on aged-animal models such as mice (Maue *et al.*, 2009) and non-human primates (Wertheimer *et al.*, 2010), however much higher life expectancy, highly individual immune properties and obvious differences in the immune system between mice and man, complicate the direct translation of these results to humans (Davis, 2008; Vallejo, 2011). The response of the human aged immune system to a challenge has been investigated so far mostly after secondary immunizations, such as influenza, tetanus or Varicella-zoster

vaccination (Hainz *et al.*, 2005; Weinberg *et al.*, 2009; McElhaney, 2011; Weinberger and Grubeck-Loebenstein, 2012). However, how the aged immune system reacts against a primary threat to a pathogenic neoantigen still remains obscure, due to the fact that studying primary acute immune responses in humans is very challenging. On the one hand, analyzing natural occurring primary infections in the elderly lacks usually knowledge about the exact time point of infection, rendering data interpretation difficult (Parsons *et al.*, 2008; Lindgren *et al.*, 2011; Lelic *et al.*, 2012; Walsh *et al.*, 2013; Blom *et al.*, 2015). On the other hand, the number of human experimental primary infections is very limited and ethically highly restricted; especially when applied to elderly study volunteers. Despite these aggravating circumstances, there is the need for more research on the immune response to primary infections in the elderly, which therefore was the main focus in the work of this dissertation.

1.3 Aging of the innate immune system

The innate immune system represents the first line of defense against infections and forms the basis for efficient initiation of a subsequent adaptive immune response. It comprises a number of different innate cell types, specialized in their functions, which have in common that they generically recognize pathogens and their activity through a multitude of invariable, germline-encoded receptors, such as Toll-like receptors (TLRs) and RIG-I-like receptors (Kumar *et al.*, 2011). Aging affects the innate immune system at various levels (Mahbub *et al.*, 2011; Shaw *et al.*, 2013), which is hallmarked by low-grade elevated levels of pro-inflammatory cytokines, clotting factors and acute phase reactants at steady state, termed “inflamm-aging” (Franceschi *et al.*, 2000; Singh and Newman, 2011). Though the exact underlying mechanisms are still incompletely understood and highly complex (Morrisette-Thomas *et al.*, 2014), this chronic pro-inflammatory basal state renders elderly individuals more prone to frailty and functional disability (Ferrucci *et al.*, 1999; Bruunsgaard *et al.*, 2003; Cohen *et al.*, 2003; Matias *et al.*, 2014). Beside this systemic phenotype, elderly individuals present also various qualitative and quantitative alterations in innate immune cell populations. Though, numbers of neutrophils are not decreased in the elderly, their functional and chemotactic capacity is compromised (Tseng and Liu, 2014). Data on other granulocyte populations in the context of aging is very sparse. Whereas eosinophils show a functional decline with age (Mathur *et al.*, 2008), basophils exhibit an age-related increased release of histamine upon stimulation (Marone *et al.*, 1986) although their absolute number decreases (Cohen *et al.*, 2013).

Monocytes are established circulating precursors for tissue macrophages and dendritic cells (DCs) and exert important innate functions such as phagocytosis, antigen presentation and cytokine production. In peripheral human blood three monocyte subsets can be distinguished: $CD14^{++}/CD16^{-}$ classical, $CD14^{++}/CD16^{+}$ intermediate and $CD14^{+}/CD16^{+}$ nonclassical monocytes (Guilliams *et al.*, 2014), with the latter two being constitutively more activated and superior in their capacity at secreting proinflammatory cytokines (Tacke and Randolph, 2006; Zimmermann *et al.*, 2010). Elderly individuals exhibit in their monocyte compartments a strong skew towards nonclassical monocytes (Sadeghi *et al.*, 1999; Seidler *et al.*, 2010; Hearps *et al.*, 2012), In addition, dysregulated monocyte function, such as decreased phagocytosis and increased basal TNFα levels (Hearps *et al.*, 2012), likely contribute to the phenotype of "inflamm-aging".

Immunological aging affects also the peripheral DC compartment (Agrawal *et al.*, 2008; Agrawal and Gupta, 2011). Plasmacytoid DCs (pDCs), which are crucial in the defense against viral infections through secreting copious amounts of anti-viral type I IFN (Liu, 2005), seem to decline in numbers and frequencies with age (Shodell and Siegal, 2002; Pérez-Cabezas *et al.*, 2007; Jing *et al.*, 2009; Garbe *et al.*, 2012; Orsini *et al.*, 2012). Additionally, studies have shown functional impairments in pDCs, such as decreased expression of TLR7 and 9 (Jing *et al.*, 2009; Garbe *et al.*, 2012) and diminished secretion of activation-induced type I and III IFNs and TNFα (Panda *et al.*, 2010; Qian *et al.*, 2011; Sridharan *et al.*, 2011). Beside pDCs, peripheral blood contains myeloid DCs (mDCs), which can be further subdivided into two subsets on the basis of CD1c and CD141 expression, termed type I and type II mDCs, respectively. Myeloid DCs are very potent antigen-presenting cells that possess the capacity to prime naive T cells with antigen of phagocytosed pathogens, thereby eliciting adaptive immunity against infections. There are conflicting reports, whether numbers of mDCs decrease or are preserved in elderly subjects (Pérez-Cabezas *et al.*, 2007; Jing *et al.*, 2009; Orsini *et al.*, 2012). However it seems that similar to pDCs, expression of certain TLRs and secretion of cytokines after stimulation is impaired in mDCs from aged individuals (Della Bella *et al.*, 2007; Panda *et al.*, 2010; Qian *et al.*, 2011), although antigen-presentation and T-cell stimulation seems to be unaffected (Steger *et al.*, 1997; Castle *et al.*, 1999). In respect to "inflamm-aging", it is of note that an increased low-grade basal expression of proinflammatory cytokines such as IL6 and TNFα has been observed in unstimulated pDCs and mDCs from the elderly (Panda *et al.*, 2010).

Natural killer (NK) cells, which are crucial modulators of anti-tumor and anti-viral immunity,

exert their function mostly through direct lysis of malign/infected cells and release of proinflammatory cytokines such as IFNγ and TNFα. Human NK cells can be broadly divided into a $CD56^{bright}$ population which predominantly secretes cytokines and $CD56^{dim}$ NK cells which have strong cytotoxic activity. In the elderly, the proportion of $CD56^{bright}$ NK cells declines, whereas $CD56^{dim}$ cells expand (Solana and Mariani, 2000). Nevertheless, overall cytotoxicity does not increase but rather decreases with age, as cytotoxic capacity on a per-cell basis diminishes (Mariani *et al.*, 1990; Hazeldine *et al.*, 2012).

Finally, there are further innate and innate-like cell subsets such as Natural killer T (NKT) cells, γδ T cells and mucosal-associated invariant T (MAIT) cells which are as well subject to immune aging as reviewed elsewhere (Peralbo *et al.*, 2007; Mocchegiani *et al.*, 2009; Roux *et al.*, 2013; Novak *et al.*, 2014; Vasudev *et al.*, 2014).

Overall, aging of innate immunity reduces the ability to properly respond to infections and to initiate the adaptive immune response. For example, reduced neutrophils and NK cell activity in the elderly are predictive of increased mortality (Niwa *et al.*, 1989; Ogata *et al.*, 2001) and age-impaired TLR function can be associated with reduced vaccine responsiveness (van Duin *et al.*, 2007).

1.4 Aging of the adaptive immune system

1.4.1 T-cell immunosenescence

T lymphocytes are major players in cellular adaptive immunity and can be broadly divided into $CD4^+$ T cells, predominantly assisting other leukocytes in immunologic processes, and $CD8^+$ T cells, which main function is to sense and destroy infected or malign cells. T cells can be classified into immunologically naive T cells and antigen-experienced memory or effector T cells according to expression of CD45RA and CCR7 (Michie *et al.*, 1992; Sallusto *et al.*, 1999). Within the naive $CD4^+$ T-cell compartment, cells having recently emigrated from the thymus, the central organ of T-cell generation, can be identified by their expression of CD31 (Kimmig *et al.*, 2002). $CD4^+$ T cells can be classified also by their functional fate and the combination of cytokines they express (Nakayamada *et al.*, 2012; Swain *et al.*, 2012). Well characterized are $CD4^+$ T helper 1 (T_H1) and T_H2 cells, which are identified by their production of IFNγ and IL4, respectively. Further subsets include $CD4^+$ T_H17 cells, which secrete IL17, and regulatory $CD4^+$ T cells (T_{reg}).

Aging strongly affects the T-cell compartment (Goronzy and Weyand, 2005). A hallmark is the

age-dependent decline of naive T cells, probably because of thymic involution and exposure to specific antigen in the periphery (Aspinall and Andrew, 2000). Thymic involution starts already in childhood and becomes clearly evident after puberty, although residual thymic activity still can be found at advanced age (Flores *et al.*, 1999; Jamieson *et al.*, 1999). Thymectomized children, who develop an altered T-cell constitution similar to those in the elderly, served as evidence for the impact of reduced thymic output on the naive T-cell compartment (Halnon *et al.*, 2005; Prelog *et al.*, 2009; Sauce *et al.*, 2009). In line with this, numbers of $CD4^+$ recent thymic emigrants ($CD4^+$ RTE) negatively correlate with chronological age (Kohler and Thiel, 2009). Whereas in young adults reduced thymic output can be compensated by naive peripheral homeostatic proliferation (Kilpatrick *et al.*, 2008), shrinkage of the naive T-cell compartment becomes more evident in individuals older than 50 years (Douek *et al.*, 1998; Goronzy and Weyand, 2005) and affects more and earlier naive $CD8^+$ T cells than naive $CD4^+$ T cells (Fagnoni *et al.*, 2000). Concomitantly, the high diversity of T-cell receptors (TCR) expressed by naive T cells declines with age, though this becomes apparent not before the last decades of life (Johnson *et al.*, 2012). From animal experiments it has been suggested, that the age-related decline in naive T-cell numbers and constrictions in the TCR diversity critically affect primary immune responses against pathogens (Yager *et al.*, 2008; Cicin-Sain *et al.*, 2010; Blackman and Woodland, 2011). It is very likely that this is true also for the human situation as elderly individuals are particularly susceptible to infections of newly arising pathogens, such as West Nile Virus or severe acute respiratory syndrome (SARS) coronavirus (Peiris *et al.*, 2003; Jean *et al.*, 2007), though this has never been investigated experimentally in humans.

The other hallmark of T-cell immunosenescence is the age-related inflation of memory and effector T cells (Fülöp *et al.*, 2013) especially in the $CD8^+$ T-cell compartment (Czesnikiewicz-Guzik *et al.*, 2008), due to the individual's immune exposure history to normal infections and particularly to chronic or persistent infections such as human cytomegalovirus (CMV), which cause specific T cells to clonally expand through repetitive stimulation (Looney *et al.*, 1999; Pourgheysari *et al.*, 2007). As a result, terminally differentiated (memory) T cells accumulate in the elderly and dominate the total T-cell compartment. Though, these cells show an exhausted phenotype, are less functional and replication senescent (Ouyang *et al.*, 2004; Fletcher *et al.*, 2005), their sheer number (Sylwester *et al.*, 2005) provides sufficient control of persistent infections, at the expense of other T cells for which they compete for resources and space (Almanzar *et al.*, 2005; Weinberger *et al.*, 2007; Brunner *et al.*, 2011). Thus age-related

memory inflation is another important mechanism impairing immunity to vaccination and infection (Goronzy *et al.*, 2001; Saurwein-Teissl *et al.*, 2002).

Beside, numerical und phenotypic alterations also numerous age-related T-cell intrinsic defects have been reported; though most of the data has been produced in animal models or stems from total T-cell populations, not discriminating quantitative aging effects (Chen *et al.*, 2013). Upon stimulation, naive T cells from aged mice showed defects in T-cell synapses, early TCR signaling events as well as cytokine secretion capacity (Poenaru *et al.*, 1990; Sadighi Akha and Miller, 2005). The only human data, describes defects in signaling downstream of the TCR in naive $CD4^+$ T cells (Li *et al.*, 2012). Hence, not only quantitative but also qualitative alterations in naive T-cell compartments might contribute to diminished induction of *de-novo* T-cell responses in aged individuals (Jiang *et al.*, 2013; Appay and Sauce, 2014).

1.4.2 Senescence of B-cell and humoral immunity

The primary purpose of the B-cell compartment is to produce antibodies, which are indispensable soluble effector molecules capable of neutralizing and opsonizing invading pathogens. Naive B cells, generated in the bone marrow, become activated after encountering cognate antigen and differentiate into acutely antibody-producing cells (also called plasmablasts), long-lived plasma cells or memory B cells, which can be discriminated by expression of surface molecules such as IgD, CD27, CD38 and CD138 (Fink, 2012). Studies in mouse models and humans revealed that during aging the B-cell compartment becomes increasingly dominated by antigen-experienced memory B cells, whereas numbers of naive B cells modestly decline (Johnson and Cambier, 2004). Concomitantly, the B cell and antibody repertoire of the elderly is less diverse (Gibson *et al.*, 2009), serum antibodies are more frequently directed against auto-antigens (Hallgren *et al.*, 1973; Mariotti *et al.*, 1992) and antibody affinities are lower than in young adults (Dunn-Walters *et al.*, 2003; Howard *et al.*, 2006). Also molecular changes, such as decreased expression of activation-induced cytidine deaminase (AID) leading to reduced *in vitro* class switch recombination (Frasca *et al.*, 2008) have been documented, though many aspects of cellular dysfunction are still unclear for human B cells (Ademokun *et al.*, 2010). Altogether, age-related defects in the B-cell compartment in combination with impairments of interacting T-cells and innate cells are associated with frailty (Gibson *et al.*, 2009) and reduced humoral immunity to various vaccinations as outlined in chapter 1.6.

1.5 Influence of persistent viral infections

Persistent viral infections have been implicated as one of the main factors driving immune aging (Brunner *et al.*, 2011). Different viruses, such as CMV, Epstein-Barr virus (EBV), Varicella-zoster virus (VZV), hepatitis B and C virus or human immunodeficiency virus (HIV) can establish chronic infections and constantly challenge the immune system of their host. As outlined before, most of these viruses are constantly present in an infected host and thereby lead to chronic antigenic stimulation resulting in clonal expansion of memory/effector T cells, which has been particularly well examined for CMV persistence (Pawelec, 2013). CMV, primarily residing in cells of the myeloid lineage where it can be intermittently reactivated (Sinclair, 2008), has a seroprevalence of approximately 60 % for the general population in industrialized countries and above 90 % in older persons (Staras *et al.*, 2006), while in developing countries it is almost 100 % (Cannon *et al.*, 2010). Similar to what is seen in aging, a clonally expanded memory T-cell pool can be observed already in CMV infected young adults, which lead to the concept that CMV persistence leads to immunosenescence already at relatively young age, or in other words CMV infection accelerates immune aging (Weinberger *et al.*, 2007; Chidrawar *et al.*, 2009; Karrer *et al.*, 2009). Additionally, CMV infection perpetually affects the NK cell and B cell compartment (Solana *et al.*, 2014; Wang *et al.*, 2014) and might be also associated with the phenotype of inflamm-aging, as increased IL6 and TNFα levels have been observed in CMV positive individuals (Trzonkowski *et al.*, 2003; Roberts *et al.*, 2010), though this has been recently challenged (Bartlett *et al.*, 2012). The entity of CMV-induced immune alterations probably negatively impacts on vaccination outcomes as it has been observed for flu vaccinations (Trzonkowski *et al.*, 2003; Moro-García *et al.*, 2012; Derhovanessian *et al.*, 2013), however no such investigations have been conducted for primary vaccinations.

EBV is another widely spread persistent virus having a seroprevalence of over 90 % in the general populations (Cohen, 2000). At latency, EBV resides in resting memory B cells (Babcock *et al.*, 1998). Although in healthy EBV carriers about 5-10 % of $CD8^+$ T cells are specific for EBV epitopes (Tan *et al.*, 1999; Hislop *et al.*, 2002), EBV has not been associated so far with strong memory inflation as seen in persistent CMV infection (White *et al.*, 2012), which might be attributed to a more dormant latency of EBV than of CMV. Further, EBV-specific T cells seem to be qualitatively comparable in young and older carriers (Cárdenas Sierra *et al.*, 2014). Instead, the B-cell compartment seems to be strongly affected by EBV persistence as it drives

clonal B cell expansion similar to what is seen during aging (Wang *et al.*, 2014), which might be disadvantageous in immune responses. Collectively, the contribution of persistent EBV infection to immune aging is less apparent than in comparison to CMV, but it is also less well understood as many aspects and consequences of EBV persistence on immunosenescence have not been investigated yet.

1.6 Vaccinations in the elderly

Vaccinations are the most effective medical intervention to prevent infectious diseases. Given the increased susceptibility and severity of infections in the elderly, preventive vaccinations seem to be the optimal strategy to promote healthy aging. However, the aforementioned age-related immune impairments, such as innate dysfunction, constrictions in the naive T-cell repertoire, memory inflation, humoral defects and co-infection with persistent viruses have to be considered when immunizing aged individuals. Indeed, numerous studies have shown that vaccination in the elderly is predominantly less effective in comparison to young adults (Weinberger and Grubeck-Loebenstein, 2012). Thus, influenza vaccination in young adults provides 65-90 % protection, whereas in the elderly only 30-50 % is achieved (Goodwin *et al.*, 2006; Nichol *et al.*, 2007). Moreover, antibody titers against tetanus and tick-borne encephalitis (TBE) virus have been shown to depend on the time since last vaccination, as well as on age (Hainz *et al.*, 2005). In this regard, pre-vaccination antibody titers are important as they positively influence vaccination outcome (Kaml *et al.*, 2006), indicating that booster vaccinations may still induce sufficient protection in aged individuals. Reduced vaccination efficiency has not only been shown for inactivated or subunit, but also for live-viral immunizations such as VZV vaccines (Levin, 2012). Although the vaccine boosts specific T cells and antibodies in the elderly (Levin *et al.*, 1992), the established $CD8^+$ T-cell immunity wanes relatively quickly (Patterson-Bartlett *et al.*, 2007), which might be the cause for the poor efficiency of only 38 % to prevent shingles in persons aged more than 69 (Oxman *et al.*, 2005). Importantly, primary immune responses to vaccination might be even more compromised, as illustrated by a 1.5 fold increased risk of non-response for Hepatitis B vaccination among older individuals (Fisman *et al.*, 2002).

With increasing life expectancy and mobility growing numbers of travelers in tropical regions are of advanced age (Hill, 2006; Gautret *et al.*, 2012). In addition, rapid aging can be observed also in developing countries, where the population is often threatened by a multitude of

tropical diseases such as Dengue fever, Japanese encephalitis or yellow fever. Therefore, vaccination in travel and tropical medicine in the elderly becomes an increasingly important topic. Yet, not many studies have examined the efficiency of travel vaccines in elderly people (Leder *et al.*, 2001), which is why such studies are absolutely needed.

1.7 Vaccination against yellow fever as a model to study primary immune responses in humans

Since its introduction 78 years ago by Theiler (Theiler and Smith, 1937), yellow fever (YF) vaccination has been administered to more than half a billion people and proven to be one of the most efficient vaccines in humans with an average seroconversion rate of >91 % and humoral protection lasting for several decades or even life-long (Poland *et al.*, 1981; Barrett *et al.*, 2007). Immunization with this live-attenuated vaccine prevents from wild type yellow fever virus (YFV) infection an otherwise highly lethal, mosquito-transmitted disease endemic to the tropical regions of Africa and South America. Infection with wild type YFV, a positive-sense, single-stranded RNA virus of the genus *Flavivirus,* family *Flaviviridae*, is in the first days characterized by abrupt fever, myalgia and chills potentially progressing in the next days to a very severe disease that include serious liver and renal dysfunction, circulatory shock and haemorrhage with a 20-50 % fatal outcome (Monath, 2008). As only symptomatic treatment is available, vaccination is the only possibility to prevent the disease.

Current YF vaccines contain the YFV-17D-204 or YFV-17DD strains, deriving from the original YFV Asibi strain through multiple passaging. By this, 48 mutations (17D) were introduced, which cause in their collectivity the attenuation (Galler *et al.*, 1998). Similar to other flaviviruses (Johnston *et al.*, 2000; Wu *et al.*, 2000; Libraty *et al.*, 2001), YFV-17D infects and activates monocytes and DCs (Liprandi and Walder, 1983; Barba-Spaeth *et al.*, 2005; Querec *et al.*, 2006; Palmer *et al.*, 2007) initiating a strong induction of innate immunity in the first days after vaccination (Gaucher *et al.*, 2008; Querec *et al.*, 2009). Furthermore, it is thought that the virus disseminates through infected DCs to lymphoid tissues, where it continues to replicate (Monath *et al.*, 2006) and finally spreads via the blood stream to its target organs such as liver, kidney and heart (Monath and Barrett, 2003). Around day 4 after vaccination a transient viremia can be detected in about 60 % of the vaccinees (Reinhardt *et al.*, 1998; Monath *et al.*, 2003, 2006), which can be found slightly delayed also in the urine (Domingo *et al.*, 2011). About one week after immunization YF-specific antibodies, $CD4^+$ T cells and $CD8^+$ T cells start to appear in peripheral blood (Reinhardt *et al.*, 1998; Martins *et al.*, 2007; Barrett

and Teuwen, 2009; Kohler *et al.*, 2012), eventually clearing the infection and giving rise to long-lasting cellular and humoral immunity. YF-specific $CD4^+$ T cells precede the cellular response peaking between day 10 and day 14 (Kohler *et al.*, 2012), whereas YF-specific $CD8^+$ T cells culminate a few days later (Miller *et al.*, 2008; Akondy *et al.*, 2009; Blom *et al.*, 2013). A remarkable finding is that in about 90 % of HLA-$A0201^+$ vaccinees up to 87 % of the YF-specific $CD8^+$ T-cell response is directed against one single immunodominant epitope in the NS4b protein, which still can be detected two years after vaccination (Akondy *et al.*, 2009).

The YF vaccine is considered to be one of the safest attenuated viral vaccines and most of the vaccinees experience only mild reactions such as low-grade fever, mild headache and myalgias (Monath *et al.*, 2005; Bae *et al.*, 2008). However, in rare cases serious YFV-related adverse events (YF-SAE) can occur, which can manifest as a neurologic (YFV-associated neurotropic disease (YEL-AND)) or viscertropic syndrome (YFV-associated viscerotropic disease (YEL-AVD)) (Monath, 2012). The incidence for YEL-AND, characterized by a post-vaccinal acute encephalitis or meningitis, has been estimated to be about 1:100.000. Risk factors that increase susceptibility to YEL-AND are very young age (<6 months) or advanced age (>60 years), reflecting an immature or age-compromised blood-brain-barrier that allows the virus to gain access to the brain or spinal cord (Monath, 2012). Usually the encephalitis is self-limiting and deaths are rare (1-2 %). This is contrasted by YEL-AVD, a syndrome closely resembling a YF-wild type infection (Hayes, 2007), which has a high case-fatality rate of about 50 %, mostly due to multiorgan failure. YEL-AVD occurs with an incidence of about 1:250.000 and unlike the live oral polio vaccine, no revertant mutations have so far been found in YF-vaccine strains that could explain a loss of attenuation (Barrett and Teuwen, 2009). Hence, unrestricted spread of YF vaccine virus and fulminant disease progression seen in YEL-AVD (Gershman *et al.*, 2012) have been attributed to unfavorable individual immunological predispositions. For example, the coincidence of a CCR5-Δ32 mutation with polymorphisms in RANTES was identified to be detrimental in a single case report of YEL-AVD (Pulendran *et al.*, 2008). Moreover, studies have shown an increased risk for developing YEL-AVD if vaccinees have thymus diseases (e.g. thymectomized individuals and thymoma patients (Eidex, 2004)), are strongly immunocompromised (e.g. HIV-infected persons with very low $CD4^+$ T-cell counts (Kengsakul *et al.*, 2002), autoimmune patients under immunosuppressive therapy (Whittembury *et al.*, 2009; Martins *et al.*, 2014) or are of advanced age (Monath *et al.*, 2005; Rafferty *et al.*, 2013). With growing numbers of elderly travelers, the 4-13 fold higher

incidence of YEL-AVD in vaccinees aged >60 years becomes particularly precarious and an individual risk-benefit analysis is indicated. It is though that mechanisms of immunosenescence are the underlying cause for the vulnerability of elderly vaccinees to develop YEL-AVD, but only two studies have so far addressed this issue. A retrospective study by Monath *et al*. compared YF-neutralizing antibodies between 2852 young and 257 elderly participants, but could not find any difference, though no YEL-AVD was observed in this study (Monath *et al.*, 2005). Another much smaller study by Roukens *et al*. observed a prolonged viremia and delayed neutralizing antibody kinetics to YF vaccination in elderly individuals, but cellular immune parameters were not assessed (Roukens *et al.*, 2011). Importantly, a comprehensive and integrated analysis of immune parameters involved in immunological aging in correlation with response kinetics to YF vaccination in aged individuals has never been conducted so far. Although this has been repeatedly suggested by several leading scientist in the field (Monath *et al.*, 2005; Pawelec *et al.*, 2010; Appay and Sauce, 2014), such a study has not been realized so far.

Beyond pure vaccinology, YF vaccination has caught the attention of basic immunologists as it provides the unique opportunity to study experimentally infections in humans (Gaucher *et al.*, 2008; Ahmed and Akondy, 2011; Kohler *et al.*, 2012). In contrast to naturally acquired infections, live-viral YF vaccination permits investigation of the immune response from the beginning of infection to the time the virus is eliminated and protective memory is formed without complications of virus persistence. Furthermore, YFV is not endemic to Europe and the US thereby true primary acute immune responses can be studied in volunteering YF vaccinees. Finally, YF vaccine is currently the only licensed live-viral primary immunization that is widely applied to adults in Europe and the US, as vaccination against smallpox has become obsolete due to its eradication. Thus, YF vaccination is an ideal experimental model infection to explore the effects of immunosenescence on a primary acute infection.

1.8 Objectives of the thesis

Elderly people have a higher susceptibility to infections and develop more often severe courses of disease, particularly when there is no pre-existing immunity, e.g. in case of newly arising pathogens such as West Nile or SARS virus. Moreover, preventive vaccinations, especially primary immunizations, have shown to be less effective and less durable in the elderly.

Mechanisms underlying the reduced immune competence to *de novo* challenges in the elderly remain largely obscure. The only data available are derived from studies with aged animals that certainly do not model the complexity and variability of human individuals. For these reasons, the primary goal of this study was to investigate and compare entire primary acute immune responses in young and elderly humans. Furthermore, we wanted to explore the impact of features of immunosenescence, such as innate dysfunction, reduced thymic activity and co-infection with persistent viruses on primary immune responses. In this respect, we intended to identify pre-immune or early immunological signatures that critically influence the acute response and the generation of protective memory. Finally, it was the goal to explore whether long-term stability of induced immunological memory is altered in the elderly.

To achieve this, we employed live-viral vaccination against yellow fever as a unique and powerful model to investigate primary acute infections in humans in an exploratory study. Furthermore, we developed a multitude of advanced, polychromatic FACS panels that allowed comprehensive characterization of human immunity before, during and after a viral challenge at close intervals in a high-throughput manner. Together with important serological parameters such as viremia and antibody response, we aimed to generate a detailed picture of YF-specific immune responses in young and aged vaccinees. Finally, we explored our data set by multivariate and correlation analysis seeking for age-related differences in the YF-specific immunity and the general immune constitution that could explain the reduced responsiveness to infections in the elderly.

2. Materials and methods

2.1 Materials

2.1.1 Consumables

Name	Vendor
BD Safety-Lok™ blood collection set 21G	BD Biosciences, Plymouth, UK
BD Vacutainer® 170 U/mL Li-Hep 10 mL	BD Biosciences, Plymouth, UK
BD Vacutainer® 1.8 mg/mL K_2EDTA 2.5 mL	BD Biosciences, Plymouth, UK
BD Vacutainer® SST™ Advance K_2EDTA 8.5 mL	BD Biosciences, Plymouth, UK
BD Trucount® tubes	BD Biosciences, San Jose, USA
Leucosep™ tubes 50 mL	Greiner, Kremsmünster, Austria
Stimulation tubes round-bottom PS 12 mL	Greiner, Kremsmünster, Austria
FACS tubes round-bottom PS 5 mL	BD Biosciences, Bedford, USA
Conical tube PP 50 mL	BD Biosciences, Durham, USA
Eppendorf tubes 0.5, 1.5, 2.0 mL Safe-Lock™	Eppendorf, Hamburg, Germany
Serological pipets 1, 5, 10, 25 and 50 mL	BD Biosciences, Franklin Lakes, USA
Pipet tips 10, 100, 1000 µL	Greiner, Kremsmünster, Austria
Riplate®, 96-deep-well, 1.2 mL, PP, v-bottom	Ritter, Schwabmünchen, Germany

2.1.2 Reagents

Reagent	Vendor/Provider	Remarks
10X BD FACS™ Lysing Solution	BD Biosciences, San Jose, USA	
10X BD FACS™ Permeabilizing Solution 2	BD Biosciences, San Jose, USA	
Fixation/Permabilization Concentrate	Ebioscience, San Diego, USA	
Fixation/Permabilization Diluent	Ebioscience, San Diego, USA	
10X Permeabilization Buffer	Ebioscience, San Diego, USA	
Beriglobin	CSL Behring, King of Prussia, USA	1 mg/mL in PBS
Bovine serum albumin (BSA)	Roche Applied Science, Penzberg, Germany	
Brefeldin A	Sigma-Aldrich, Munich, Germany	5 mg/mL in 70 % ethanol
BD GolgiStop™ (Monensin)	BD Biosciences, San Jose, USA	
Anti-human CD28 NA/LE	BD Biosciences, San Jose, USA	1 mg/mL in PBS
DMSO	Sigma-Aldrich, Munich, Germany	
DAPI	Roche, Basel, Switzerland	1 µg/mL in PBS
Casy®ton	Roche, Basel, Switzerland	
EL (Erythrocyte Lysis) buffer	Qiagen, Hilden, Germany	
Human AB serum	Lonza, Basel, Switzerland	Heat-inactivated at 56 °C
Live/Dead® Fixable Aqua	Life Technologies, Carlsbad, USA	Solved in 500 µL DMSO
Live/Dead® Fixable Red	Life Technologies, Carlsbad, USA	Solved in 500 µL DMSO and pre-diluted 1:100 before usage
Lymphocyte separation medium LSM 1077	PAA, Pasching, Austria	
Yellow fever vaccine (Stamaril®)	Sanofi Pasteur MSD, Berkshire, UK	Used for vaccination
Yellow fever vaccine (RKI)	Robert Koch-Institute, Berlin Germany	Used for stimulation 1 human dose solved with 150 µL RPMI/AB
NS4b 9mer	JPT peptide technologies, Berlin, Germany	LLWNGPMAV 0.25 µg/µL in 50 % DMSO / 50 % PBS
Staphylococcus enterotoxin B (SEB)	Sigma-Aldrich, Munich, Germany	1.5 µg/µL in PBS
Toxic shock syndrome toxin 1 (TSST1)	Sigma-Aldrich, Munich, Germany	1.0 µg/µL in PBS

CMV pp65 peptide pool	JPT peptide technologies, Berlin, Germany	Swiss Prot ID.: P06725, 138 15mers, 11 aa overlap, 0.25 µg/µL per peptide solved in 50 % DMSO / 50 % PBS
CMV IE-1 peptide pool	JPT peptide technologies, Berlin, Germany	NCBI seq.: CAA35325.1, 120 15mers, 11 aa overlap, 0.25 µg/µL per peptide solved in 50 % DMSO / 50 % PBS
EBV EBNA1 peptide pool	JPT peptide technologies, Berlin, Germany	NCBI seq.: YP401677, 158 15mers, 11 aa overlap 0.25 µg/µL per peptide solved in 50 % DMSO / 50 % PBS
EBV BZLF1 peptide pool	JPT peptide technologies, Berlin, Germany	NCBI seq.: YP_401673, 59 15mers, 11 aa overlap 0.25 µg/µL per peptide solved in 50 % DMSO / 50 % PBS
Penicillin	Merckmillipore, Darmstadt, Germany	
Streptomycin	Merckmillipore, Darmstadt, Germany	
Gibco® RPMI 1640 GlutaMAX™ cell culture medium	Life Technologies, Carlsbad, USA	
10X PBS pH 7.2	Life Technologies, Carlsbad, USA	
EDTA	Merckmillipore, Darmstadt, Germany	
BD FACSFlow™	BD Biosciences, San Jose, USA	
MACSQuant Storage Solution	Miltenyi, Bergisch-Gladbach, Germany	
MACSQuant Washing Solution	Miltenyi, Bergisch-Gladbach, Germany	
MACSQuant Running Buffer	Miltenyi, Bergisch-Gladbach, Germany	
SPHERO™ Rainbow Calibration Particles	BD Biosciences, San Jose, USA	
Millipore H_2O		

2.1.3 Kits

Kit name	**Vendor**
Anti-Flavivirus mosaic IIFA	Euroimmun, Lübeck, Germany
Anti-Yellow fever virus IIFA with IgM, IgG, IgA	Euroimmun, Lübeck, Germany
CMV IgG-Elisa PCS	Medac, Wedel, Germany
EBV IgG-Elisa PCS	Medac, Wedel, Germany
QIAamp viral RNA minikit	Qiagen, Hilden, Germany
QuantiTect Virus + ROX Vial kit	Qiagen, Hilden, Germany

2.1.4 Solutions

Solution	Preparation
1X PBS	10X PBS diluted 1:10 in H_2O
1X PBS/BSA	1X PBS + 5 g/L BSA
20 nM EDTA	EDTA solved in PBS
1X BD FACS™ Lysing Solution	10X BD FACS™ Lysing Solution 1:10 in H_2O
1X BD FACS™ Permeabilizing Solution 2	10X BD FACS™ Permeabilizing Solution 2 1:10 in H_2O
1X FoxP3 fixation buffer	Fixation/Permabilization Concentrate diluted 1:4 with Fixation/Permabilization Diluent
1X FoxP3 permeabilization buffer	10X Permeabilization Buffer diluted 1:10 in H_2O
RPMI/AB	RPMI + 10 % AB serum + 100 U/mL Penicillin + 0.1 mg/mL Streptomycin

2.1.5 Software

Software	Vendor/Provider
BD FACSDiva 6	BD Biosciences, San Jose, USA
MACSQuantify software	Miltenyi, Bergisch-Gladbach, Germany
Flowjo 9.7.x	Tree Star, Ashland, USA
SPICE 5.3	Joshua Nozzi & Mario Roederer, Vaccine Research Center, NIAID, NIH
Pestle 1.7	Mario Roederer, Vaccine Research Center, NIAID, NIH
GraphPad Prism 5.x	GraphPad Software, La Jolla, USA
R 3.02	R Foundation for Statistical Computing, Vienna, Austria

2.1.6 Equipment

Device	Vendor
CO_2 incubator Innova CO-170	New Brunswick Scientific, Enfield, USA
BD™ LSR II flow cytometer	BD Biosciences, San Jose, USA
MACSQuant® analyzer	Miltenyi, Bergisch-Gladbach, Germany
Eppendorf Research® pipets	Eppendorf, Hamburg, Germany
Pipetboy® pipet controller	Integra, Zizers, Switzerland
HERAsafe® biosafety cabinet	Heraeus, Hanau, Germany
Centrifuges 5804R and 5810R	Eppendorf, Hamburg, Germany
Centrifuges Microfuge® 22, Allegra® X-15R and X22	Beckman Coulter, Krefeld, Germany
Vortex Mixer	VWR International, Darmstadt, Germany
CASY Model TTC cell counter	Roche Applied Science, Penzberg, Germany
Nalgene® Test tube rack, 16 mm for stimulation	Thermo Fisher Scientific, Waltham, USA
Vacuum pump BVC 21	Vacuubrand, Wertheim, Germany
ELISA processor BEP III	Siemens, Erlangen, Germany
Sysmex XE-5000	Sysmex, Kobe, Japan

2.1.7 Optical configurations of flow cytometers

Table 2.1: Laser and optical filter settings on LSR II

LSR II		WB leukocyte counting panel		Dendritic cell panel		Recent thymic emigrant panel		T-cell activation panel		WB CD4$^+$ T-cell stim. panel & PBMC stim. panel	
Laser line	Detector name	Long pass	Band pass	Long pass	Band pass	Long pass	Band pass	Long pass	Band pass	Long pass	Band pass
Blue 488 nm	SSC	n.a.	488/10	n.a.	488/10	n.a.	488/10	n.a.	488/10	n.a.	488/10
	B525	505	525/50	505	525/50	505	525/50	505	525/50	505	525/50
	B685	685	710/40	685	710/40	685	710/40	685	710/40	685	710/40
Yellow-green 561 nm	YG582	n.a.	582/15	n.a.	582/15	n.a.	582/15	n.a.	582/15	n.a.	582/15
	YG610	600	610/20	600	610/20	600	610/20	600	610/20	600	610/20
	YG670	630	670/14	630	670/14	630	670/14	630	670/14	630	670/14
	YG710	685	710/50	685	710/50	685	710/50	685	710/50	685	710/50
	YG780	755	780/60	755	780/60	755	780/60	755	780/60	755	780/60
Red 640 nm	R660	n.a.	660/13	n.a.	660/13	n.a.	660/13	n.a.	660/13	n.a.	660/13
	R720	685	720/30	685	720/30	685	720/30	685	720/30	685	720/30
	R780	755	780/60	755	780/60	755	780/60	755	780/60	755	780/60
Violet 405 nm	V450	n.a	450/50	n.a	450/50	n.a	450/50	n.a	450/50	n.a	450/50
	V525	505	525/50	505	525/50	505	525/50	505	525/50	505	525/50
	V610	595	610/20	595	610/20	570	575/25	570	575/26	595	610/20
	V710	685	710/50	650	661/20	595	610/20	595	610/20	650	661/20
	V780	755	780/60	755	780/60	650	661/20	755	780/60	755	780/60

Table 2.2: Laser and optical filter settings on MACSQuant

MACS Quant		WB leukocyte counting panel	B cell panel	NK cell panel
Laser line	Detector name	Filter	Filter	Filter
Blue 488 nm	SSC	488/10	488/10	488/10
	B525	525/50	525/50	525/50
	B585	585/40	585/40	585/40
	B655	655LP	655LP	655LP
	B750	750LP	750LP	750LP
Red 633 nm	R655	655LP	655LP	655LP
	R750	750LP	750LP	750LP
Violet 405 nm	V450	450/50	450/50	450/50
	V525	525/50	525/50	525/50

2.2 Methods

2.2.1 Vaccination and study cohort

Approval for the study was obtained from the ethics committee of the Faculty of Medicine - Charité, Humboldt University Berlin. After informed consent and prior vaccination sera of 26 volunteers were analyzed for potentially cross-reactive antibodies against flaviviruses by a Euroimmun anti-Flavivirus mosaic indirect immunofluorescence assay (IIFA) (Chap. 2.2.2). Two donors showed tick-borne encephalitis virus-specific antibodies and hence were omitted from analysis. Another donor retrospectively demonstrated unusual immunological features (high steady state T-cell activation) preventing proper evaluation and was therefore excluded from analysis as well. The remaining 23 volunteers were assigned into two age groups: “Young” (11 donors, 20-30 years, median age 26 years, 5 female/6 male) and “Elderly” (12 donors, 55-70 years, median age 60 years, 5 female/7 male). The upper age limit for participation was 70 years set by the ethical approval. Health status and current medication was assessed by questionnaire to exclude individuals with acute illness, a history of cancer, autoimmune disease, allergies or innate or acquired immunosuppression. Two of the elderly subjects were on medication for hypertension, 1 for Diabetes mellitus type II and 1 for benign prostatic hyperplasia. Immunization was performed by a single subcutaneous injection of YFV-17D vaccine (Stamaril®). Any adverse reactions (erythema, swelling, myalgia, fever) were documented. Fifty mL of venous blood was drawn immediately before vaccination (day 0) and at eight further time-points (day 2 (only donors 15 to 28), 4, 7, 10, 14, 17, 21 and 28) after vaccination into sodium heparin, serum gel or EDTA tubes. To study long-term YF-specific memory formation and stability, we collected in each case 50 mL of venous blood from 19 of 23 vaccinees re-invited 33 (donor 1 - 6), 30 (donor 8 - 14) or 20 (donor 15 - 27) months after vaccination. Heparinized blood was processed and analyzed directly. Sera were stored at -80 °C until use.

2.2.2 Anti-Flavivirus mosaic indirect immunofluorescence assay

In order to exclude individuals with pre-existing, potentially cross-reactive antibodies against flaviviruses, an anti-Flavivirus mosaic IIFA specific for YF wildtype and vaccine virus, Japanese encephalitis virus, dengue virus (type 1-4), tick-borne encephalitis virus and West Nile virus was performed according to the manufacturer’s protocol by the group of Prof. Niedrig,

Robert Koch-Institute (RKI), Berlin. Briefly, sera from day 0 were diluted 1:10 and applied to the reaction fields of a reagent tray. Then, a microscope slide containing the flavivirus mosaic biochips was placed upside down on the reagent tray. After 30 min of incubation at RT and a PBS/Tween buffer washing step, biochips were incubated with FITC-labeled anti-human IgG. Titers of pre-existing flavivirus-crossreactive antibodies were determined by fluorescence microscopy according to the Euroimmun standard procedure based on fluorescence intensity. Titers of ≥1:10 were considered positive.

2.2.3 Detection of serum viremia with quantitative real-time PCR (RT-PCR)

Sera were tested for the presence of YFV-17D genomes at days 0, 2, 4, 7 and 10 after vaccination by quantitative RT-PCR as described elsewhere (Domingo *et al.*, 2012) in collaboration with the group of Prof. Niedrig, RKI, Berlin. In short, viral RNA was extracted with a QIAamp viral RNA minikit. Next, a quantitative RT-PCR was performed with the QuantiTect Virus + ROX Vial kit, using YF-specific primers and 2 µL of viral RNA in a 25 µL reaction. In order to standardize the measurement, a serial dilution of in-vitro generated viral RNA of known concentration was simultaneously run. The assay has a limit of detection of 0.8 plaque forming units per mL (95 % CI).

2.2.4 Assessment of YF-specific serum response

We examined the YF-specific IgM, IgG and IgA response at all study days in collaboration with the group of Prof. Niedrig using an anti-Yellow fever virus IIFA. For IgM and IgA analysis, sera were pre-diluted 1:10 and for IgG analysis 1:100. Then, samples were incubated with anti-Yellow fever virus biochips as described in chap. 2.2.2 After this, biochips were incubated either with FITC-labeled anti-human IgM, IgA or IgG. Antibody titers were determined by fluorescence microscopy according to the Euroimmun standard procedure, rating fluorescence intensities in levels from 0 to 5. In a 1:10 dilution, level 1 was rated as an antibody titer of 1:10, level 2 as 1:32, level 3 as 1:100 and level 4 and 5 were rated as more than 1:100. Accordingly, in a 1:100 dilution, level 1 was rated as 1:100, level 2 as 1:320 and so on. Titers above 1:10 (IgM, IgA) or 1:100 (IgG) were considered positive.

2.2.5 Plaque reduction neutralization test (PRNT90)

PRNT90 assays of stored sera from all study time-points were carried out by the group of Prof. Niedrig, according to the protocol established by Reinhardt (Reinhardt *et al.*, 1998). Briefly,

two-fold dilutions of sera ranging from 1:10 to 1:320 were prepared and mixed with YFV-17D in DMEM. After 1 h of incubation at 37 °C, $1.2x10^5$ PS cells were added to each dilution and the mixes (400 µL) were plated into a 24 well plate. After 4 h of incubation at 37 °C in the incubator, cultures were overlaid with 400 µL high-viscous carboxylmethyl cellulose DMEM. Plaques were counted after 4 days of incubation at 37 °C, 3.7 % formaldehyde fixation and naphthalene black staining. The 90 % neutralization titers were calculated according to Reed and Muench (Reed and Muench, 1938)

2.2.6 Complete blood count

Complete blood counts were performed by Labor Berlin – Charité Vivantes GmbH, Berlin, Germany for vaccinees 8 – 28. For this freshly collected EDTA blood was analyzed by the automated cell counter Sysmex XE-5000 for absolute whole blood number of leukocytes, lymphocytes, monocytes, basophils, eosinophils and neutrophils.

2.2.7 CMV and EBV serology

Specific IgG responses against CMV and EBV were measured in day 0 sera of all vaccinees in cooperation with Labor Berlin – Charité Vivantes GmbH, Berlin, Germany. Sera were automatically analyzed with the CMV or EBV IgG-Elisa PCS on an ELISA processor BEP III.

2.2.8 Isolation of peripheral blood mononuclear cells (PBMC)

PBMCs were isolated from freshly drawn heparinized whole blood in 50 mL Leucosep tubes filled with 14 mL Ficoll-Hypaque (LSM 1077) according to the manufacturer's protocol. In brief, blood was diluted 1:1 with PBS/BSA and transferred to Leucosep tubes. Following 10 min of centrifugation at 1000 g at RT, supernatant above the leukocyte layer was removed and PBMCs were recovered by pouring them into a fresh 50 mL tube. Next, PBMCs were washed twice with PBS/BSA by centrifugation at 490 g, 10 min 4 °C and 180 g, 15 min, 4 °C, respectively. Finally, a sample of 10 µL of PBMCs was counted with a CASY Model TTC cell counter equipped with a 150 µm capillary and with exclusion of cell debris and dead cells. Finally, PBMCs were re-suspended at a concentration of $1x10^7$ cells/mL with PBS/BSA and freshly used in subsequent assays.

2.2.9 Flow cytometric analysis

2.2.9.1 Analysis of absolute frequencies of leukocyte populations in peripheral blood

To determine absolute counts of the analyzed leukocyte populations in freshly drawn peripheral blood, 50 µL of heparinized whole blood was incubated for 15 min at RT with 50 µL fluorescence-labeled monoclonal antibody cocktail (Table 2.3). Exact volumes were achieved by reverse pipetting. Staining was stopped and erythrocytes lysed by adding 500 µL Buffer EL. Following 15 min incubation at 4 °C, samples were immediately analyzed on a MACSQuant or LSR II flow cytometers without prior centrifugation. The optical configurations are listed in table 2.1 and 2.2. The cell trigger on the instruments was set to the CD45 channel. An example gating strategy is depicted in Fig. 2.1. By analyzing a defined sample up-take volume (350 µL), MACSQuant gave direct cell counts for the labeled populations. Cell counts measured on the LSR II were based on the use of BD TruCount™ tubes containing a defined number of fluorescent quantification beads. Counts of major leukocyte populations were used for calculations of any given cell subpopulation determined by other FACS panels.

Table 2.3: Antibody cocktails for whole blood leukocyte counting

Name	Fluorochrome	Clone	Isotype	Vendor/Provider	Dilution
Whole blood leukocyte counting panel (Round 1 & 2, LSR II)					
CD3	PacBlue	OKT3	mouse IgG2a	Biolegend	1:50
CD8	efluor605	RPA-T8	mouse IgG1	Ebioscience	1:50
CD14	FITC	M5E2	mouse IgG2a	BD Biosciences	1:50
CD4	PerCpCy5.5	OKT4	mouse IgG2b	Biolegend	1:50
CD19	PE	BU12	mouse IgG1	in house DRFZ	1:200
CD56	PECy7	NCAM16.2	mouse IgG2b	BD Biosciences	1:50
CD11c	APC	MJ4-27G12	mouse IgG2b	Miltenyi	1:20
CD45	APCCy7	HI30	mouse IgG1	Biolegend	1:20
Beriglobin	n.a.	n.a.	n.a.	Aventis	1:50
Whole blood leukocyte counting panel (Round 3, MACSQuant)					
CD14	Vioblue	TÜK4	mouse IgG2a	Miltenyi	1:100
CD45	Viogreen	5B1	mouse IgG2a	Miltenyi	1:20
BDCA2	FITC	AC144	mouse IgG1	Miltenyi	1:100
CD56	PE	AF12-7H3	mouse IgG1	Miltenyi	1:40
CD8	PerCp	BW135/80	mouse IgG2a	Miltenyi	1:10
CD19	PEVio770	LT19	mouse IgG1	Miltenyi	1:20
CD4	APC	VIT4	mouse IgG2a	Miltenyi	1:20
CD3	APCH7	SK7	mouse IgG1	BD Biosciences	1:50
Beriglobin	n.a.	n.a.	n.a.	Aventis	1:50

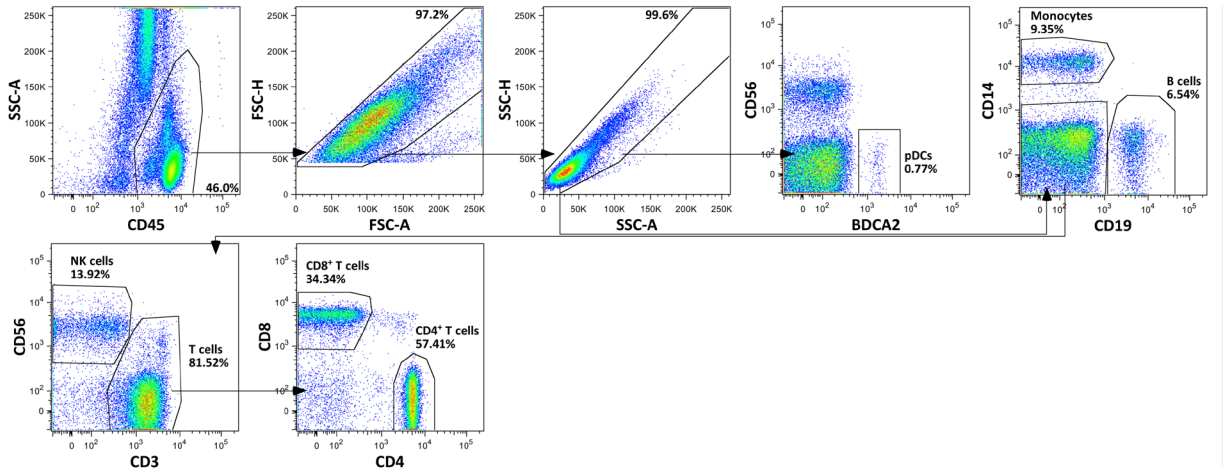


Figure 2.1: Gating strategy for the whole blood counting panel measured on MACSQuant.
Cells from lysed whole blood were gated on CD45. After exclusion of cell aggregates, pDCs were directly determined by expression of BDCA2. Alternatively monocytes ($CD14^+$) and B cells ($CD19^+$) were identified. Cells being neither a monocyte nor a B cell were further distinguished into T cells ($CD3^+$) and NK cells ($CD56^+$). T cells were sub-divided into $CD4^+$ and $CD8^+$ T cells. Calculations of whole blood cell counts from other panels based on measured events of populations determined in this panel. Depicted is donor 15 (young) at day 0.

2.2.9.2 Dendritic cell panel

Innate cell subsets, such as monocytes and DCs were assessed by a 12-color FACS panel on days 0, 4, 7, 10, 14, 17, 21 and 28. For this, $5x10^6$ PBMCs of each donor re-suspended in 60 µL PBS/BSA were stained with 40 µL fluorescence-labeled monoclonal antibody cocktail (Table 2.4) for 15 min at RT and in the dark in FACS tubes. Then, cells were washed once with PBS/BSA (490 g, 10 min, 4 °C) and analyzed on a LSR II flow cytometer (optical filter setting see table 2.1). Prior acquisition DAPI was added at concentration of 0.01 µg/mL. Figure 2.2 illustrates the gating strategy for this panel. We identified the following innate cell types: plasmacytoid DCs, myeloid DCs type I and type II, classical monocytes, intermediate monocytes, nonclassical monocytes and basophils. Absolute whole blood counts of these cell populations were calculated based on events acquired in the $CD14^{++}$ gate.

Table 2.4: Antibody cocktail for dendritic cell panel

Name	Fluorochrome	Clone	Isotype	Vendor	Dilution
Dendritic cell panel (LSR II)					
CD56	BV421	HCD56	mouse IgG1	Biolegend	1:100
CD19	V500	HIB19	mouse IgG1	BD Biosciences	1:100
CD3	V500	SP34-2	mouse IgG1	BD Biosciences	1:50
CD14	efluor605	61D3	mouse IgG1	Ebioscience	1:50
CD123	efluor650	6H6	mouse IgG1	Ebioscience	1:50
BDCA2	FITC	AC144	mouse IgG1	Miltenyi	1:100
BDCA1 (CD1c)	PerCpCy5.5	L161	mouse IgG1	Biolegend	1:50
BDCA3 (CD141)	PE	AD5-14H12	mouse IgG1	Miltenyi	1:10
HLA-DR	PECy7	L243	mouse IgG2a	Biolegend	1:100
CD11c	APC	MJ4-27G12	mouse IgG2b	Miltenyi	1:20
CD16	Alexa700	3G8	mouse IgG1	Biolegend	1:100
CD45	APCH7	2D1	mouse IgG1	BD Biosciences	1:200
Beriglobin	n.a.	n.a.	n.a.	Aventis	1:50

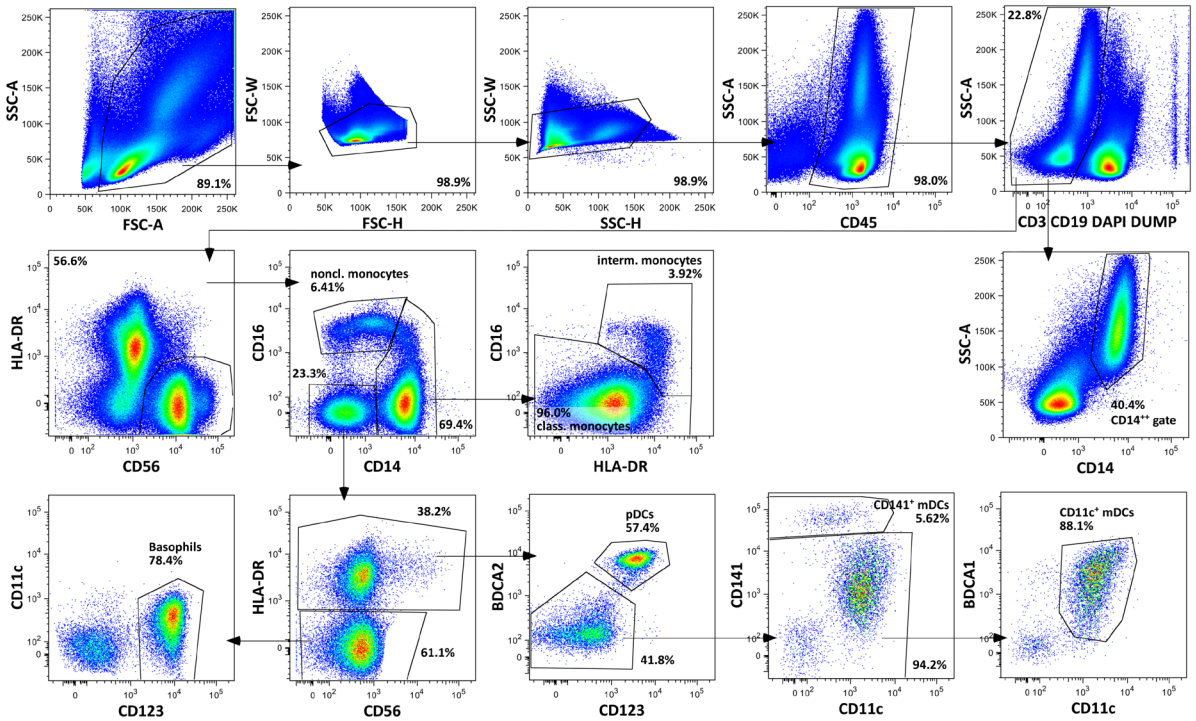


Figure 2.2: Gating strategy for the dendritic cell panel.

For analysis of innate cell subsets from PBMCs, a broad gate was used for the pre-selection of leukocyte scatter. After exclusion of cell aggregates, $CD45^+/CD3^-/CD19^-$/live cells were selected and NK cells ($HLA\text{-}DR^-/CD56^+$) excluded. From remaining cells monocytes subsets were determined by gating on CD14 and CD16. Intermediate monocytes were distinguished from classical monocytes by their increased expression of HLA-DR. Cells that did not belong to any monocyte subset were further analyzed for their HLA-DR expression. $HLA\text{-}DR^-/CD123^+$ cells could be identified as basophils, whereas $HLA\text{-}DR^+$ cells were DCs. Within the DC population, pDCs were defined as $BDCA2^+/CD123^+$. DCs, negative for BDCA2 and CD123 were assessed for their expression of CD11c (expressed by all mDCs) and CD141 (positive on type 2 mDCs (mDC2) only). Lastly, $CD141^{neg}$ cells were examined for their expression of BDCA1 defining type 1 mDCs (mDC1). Depicted is donor 15 (young) at day 0.

2.2.9.3 Recent thymic emigrant (RTE) panel

RTEs, a subset of naive $CD4^+$ T cells, were assessed on day 0 by an 11-color FACS panel. The general staining procedure was similar to the DC panel staining, except for the incubation temperature, which was 37 °C. The antibody cocktail used is listed in table 2.5. Optical filter settings on LSR II are shown in table 2.1. Figure 2.3 illustrates the gating strategy for RTEs. Absolute whole blood counts of cell populations were calculated based on events acquired in the $CD4^+$ gate. This panel included also an anti-HLA-A2 staining, which allowed discrimination of HLA-A0201 positive and negative individuals.

Table 2.5: Antibody cocktail for RTE panel

Name	Fluorochrome	Clone	Isotype	Vendor	Dilution
Recent thymic emigrant panel (LSR II)					
CD45RO	PacBlue	UCHL1	mouse IgG2a	Biolegend	1:50
CD8	BV570	RPA-T8	mouse IgG1	Biolegend	1:50
CD45RA	efluor605	HI100	mouse IgG2b	Ebioscience	1:100
CD3	efluor650	OKT3	mouse IgG2a	Ebioscience	1:50
HLA-A2	FITC	BB7.2	mouse IgG2b	Biolegend	1:200
CCR7	PerCpCy5.5	G043H7	mouse IgG2a	Biolegend	1:50

Name	Fluorochrome	Clone	Isotype	Vendor	Dilution
CD31	PE	WM59	mouse IgG1	Biolegend	1:100
CD27	PECy7	M-T271	mouse IgG1	BD Biosciences	1:100
CD62L	APC	DREG-56	mouse IgG1	Biolegend	1:50
CD4	Alexa700	OKT4	mouse IgG2b	Biolegend	1:200
Beriglobin	n.a.	n.a.	n.a.	Aventis	1:50

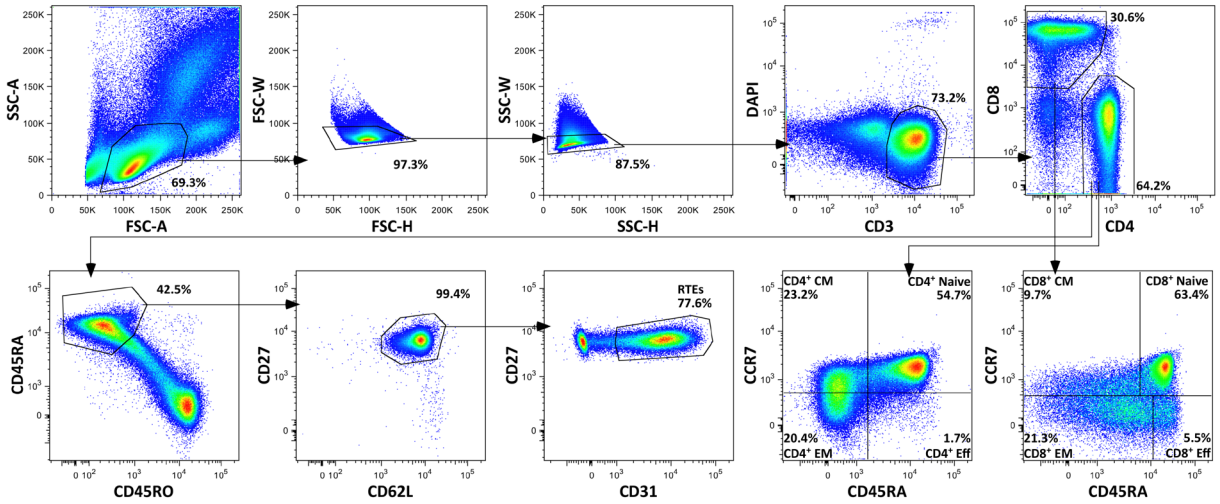


Figure 2.3: Gating strategy for RTE panel.
RTEs and other $CD4^+$ and $CD8^+$ T-cell subsets were analyzed in PBMCs by gating on lymphocytes by light scatter and excluding cell aggregates and dead cells. T cells were identified by CD3 expression and further sub-divided into $CD4^+$ and $CD8^+$ T cells. For determination of RTEs, $CD4^+$ T cells were sequentially gated on several markers indicating the naivety, such as CD45RA, CD27 and CD62L. From these naive $CD4^+$ T cells, RTEs could be assessed by their expression of CD31. For determination of T-cell subsets such as naive, central-memory (CM), effector-memory (EM) and effector (Eff) T cells, $CD4^+$ or $CD8^+$ T cells were investigated for combinatorial expression of the markers CD45RA and CCR7, respectively. Depicted is donor 15 (young) at day 0.

2.2.9.4 B-cell panel

Various B-cell subsets were examined at days 0, 4, 7, 10, 14, 17, 21 and 28 after vaccination. For this, $5x10^6$ PBMCs of each donor were transferred into a 96-deep well plate. Then, PBMCs were spun down at 490 g, 10 min, 4 °C and supernatant was completely removed. Next, each cell pellet was re-suspended in 90 µL PBS/BSA and 22 µL of fluorescence-labeled monoclonal antibody cocktail (Table 2.6) was added. This surface staining was performed at RT for 15 min. After 5 min of incubation 1 µL of L/D Aqua was supplemented to each well and the plate was shortly vortexed. Staining was stopped by adding 800 µL PBS/BSA, then cells were centrifuged 10 min, 310 g, 4 °C and supernatant was removed. Next, surface stained PBMCs were fixed by re-suspension in 800 µL 1X BD FACS Lysing solution for 10 min at RT. Fixed cells were directly spun down (10 min, 490 g, 4 °C), supernatant discarded and 300 µL 1X BD Perm 2 solution added to each well in order to permeabilize the samples. The reaction was stopped by adding 700 µL PBS/BSA, followed by a centrifugation step (10 min, 490 g, 4 °C). Fixed and permeabilized PBMCs were washed again with 1 mL PBS/BSA (10 min, 490 g, 4 °C) and

supernatant was discarded. Finally, each cell pellet was re-suspended in 100 µL PBS/BSA and intracellularly stained 30 min at RT for Ki-67 expression. After a washing step with 900 µL PBS/BSA and final centrifugation (10 min, 490 g, 4 °C), each cell pellet was re-suspended in 400 µL PBS/BSA and automatically acquired on a MACSQuant flow cytometer (uptake volume 300 µL) with an optical configuration listed in table 2.2. An exemplary gating is shown in fig. 2.4. Absolute whole blood counts of cell populations were calculated based on events acquired in the $CD19^+$ gate.

Table 2.6: Antibody cocktail for B-cell panel

Name	Fluorochrome	Clone	Isotype	Vendor	Dilution
B-cell panel (MACSQuant) – surface staining					
CD3	V500	SP34-2	mouse IgG1	BD Biosciences	1:50
CD14	V500	M5E2	mouse IgG2a	BD Biosciences	1:100
IgD	FITC	IA6-2	mouse IgG2a	Biolegend	1:20
CD19	PE	BU12	mouse IgG1	in house DRFZ	1:200
CD20	PerCp	LT20	mouse IgG1	Miltenyi	1:20
CD38	PECy7	HIT2	mouse IgG1	Biolegend	1:100
CD27	APC	L128	mouse IgG1	BD Biosciences	1:20
HLA-DR	APCH7	G46-6	mouse IgG2a	BD Biosciences	1:100
Beriglobin	n.a.	n.a.	n.a.	Aventis	1:50
B-cell panel (MACSQuant) – intracellular staining					
Ki-67	V450	B56	mouse IgG1	BD Biosciences	1:50

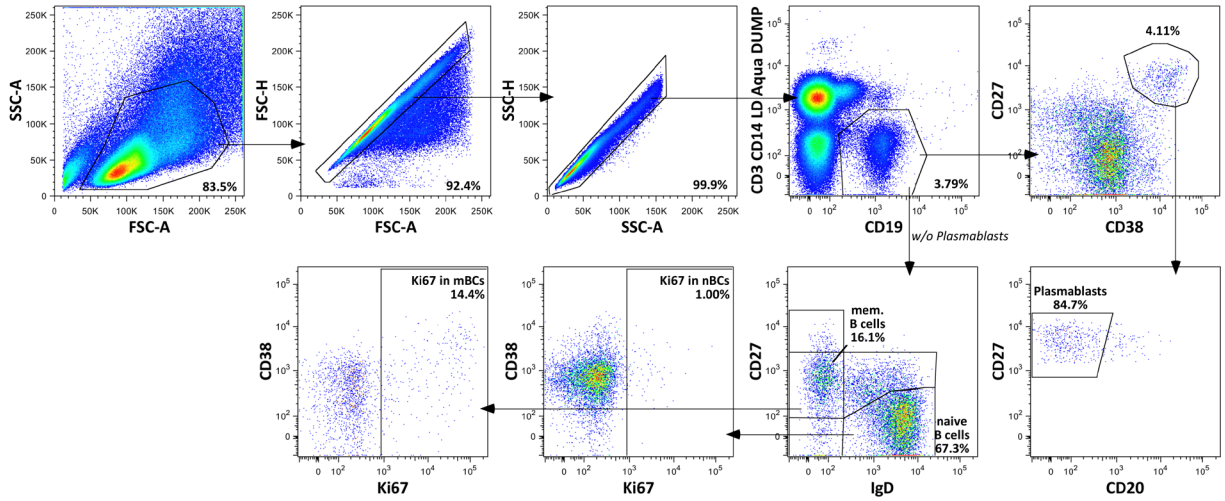


Figure 2.4: Gating strategy for the B-cell panel.

B-cell subsets and their proliferation status were assessed in fixated PBMCs by loosely gating on lymphocytes by light scatter and excluding cell aggregates and dead cells. Then B cells were identified by gating on $CD19^+$ cells omitting cells expressing CD3 and CD14. A relatively large CD19 gate was used to prevent unintended trimming of plasmablast having a low CD19 expression. Plasmablasts were identified in $CD19^{low/+}$ cells by the high co-expression of CD38 and CD27. To ensure correct plasmablast assignment, $CD38^+/CD27^+$ cells were checked for absence of CD20 expression. Reported percentages of plasmablasts always refer to $CD19^{low/+}$ B cells. Other B-cell subsets were determined from plasmablast negative and $CD19^{low/+}$ B cells (Boolean gating). Expression of IgD and CD27 allowed for differentiation of memory ($IgD^-/CD27^+$) and naive ($IgD^+/CD27^-$) B cells, which were further analyzed for their proliferation status by Ki-67 expression. Depicted is donor 15 (young) at day 0.

2.2.9.5 NK-cell panel

NK cells were assessed at days 0, 4, 7, 10, 14, 17, 21 and 28 after vaccination. The protocol used was similar to the B cell panel staining procedure. The antibody cocktail is listed in table 2.7. Automated acquisition was done on a MACSQuant (uptake volume 300 µL) with an optical configuration listed in table 2.2. An exemplary gating is shown in fig. 2.5. Absolute whole blood counts of cell populations were calculated based on events acquired in the $CD56^+$ gate.

Table 2.7: Antibody cocktail for NK-cell panel

Name	Fluorochrome	Clone	Isotype	Vendor	Dilution
NK-cell panel (MACSQuant) – surface staining					
CD56	BV421	HCD56	mouse IgG1	Biolegend	1:100
CD3	V500	SP34-2	mouse IgG1	BD Biosciences	1:50
CD14	Viogreen	TÜK4	mouse IgG2a	Miltenyi	1:20
CD62L	PE	DREG-56	mouse IgG1	Biolegend	1:20
CD27	PerCp	O323	mouse IgG1	Biolegend	1:150
CD69	PECy7	FN50	mouse IgG1	Biolegend	1:200
CD57	Alexa647	HCD57	mouse IgM	Biolegend	1:80
CD16	APCH7	3G8	mouse IgG1	BD Biosciences	1:200
Beriglobin	n.a.	n.a.	n.a.	Aventis	1:50
NK-cell panel (MACSQuant) – intracellular staining					
Ki-67	Alexa488	B56	mouse IgG1	BD Biosciences	1:100

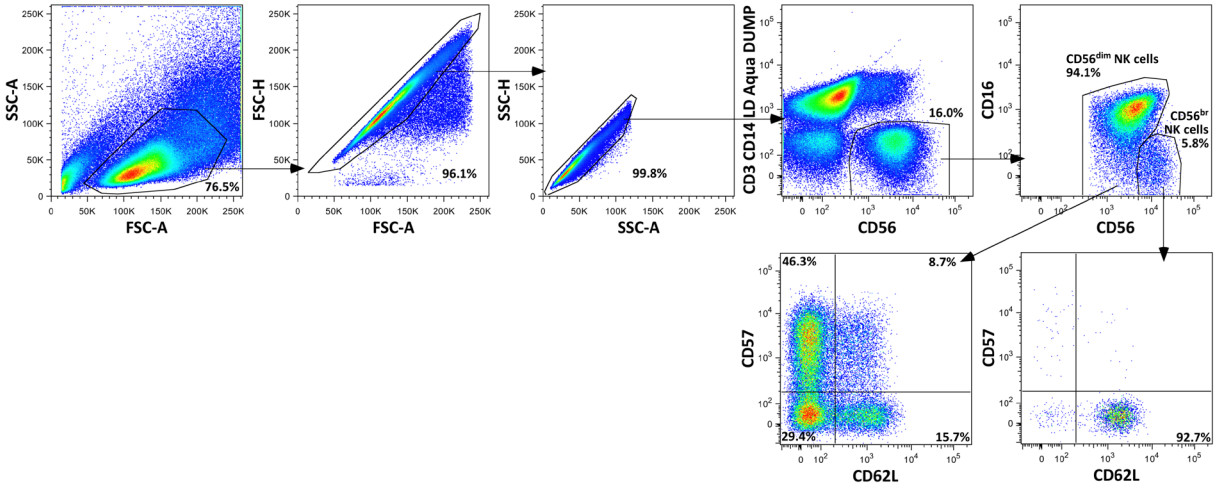


Figure 2.5: Gating strategy for NK-cell panel

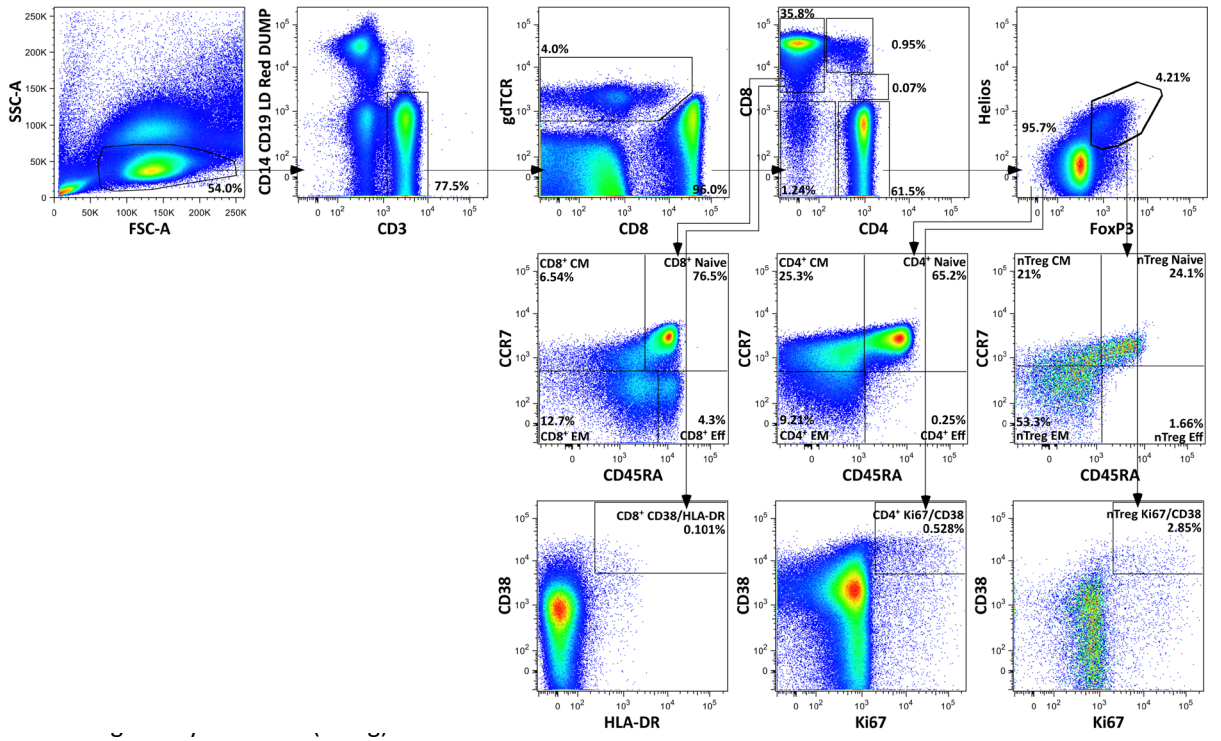
NK-cell subsets and their proliferation status were assessed in fixated PBMCs by gating on lymphocytes by light scatter and excluding cell aggregates and dead cells. Then, NK cells were identified by gating on $CD56^+$ cells omitting cells expressing CD3 and CD14. Next, $CD56^{bright}$ NK cells were distinguished from $CD56^{dim}$ NK cells by the help of CD16, that is higher expressed in the latter. $CD56^{bright}$ NK cells were almost exclusively $CD62L^+$ and $CD57^-$, whereas $CD56^{dim}$ NK cells could be sub-divided into four subsets by both markers. Depicted is donor 15 (young) at day 0.

2.2.9.6 T-cell activation panel

Diverse T-cell subsets and their activation and proliferation status were analyzed at days 0, 2, 4, 7, 10, 14, 17, 21 and 28 after vaccination. This included also measurement of regulatory T-cells in donors 15 to 28. For this, $5x10^6$ PBMCs of each donor re-suspended in 100 µL PBS/BSA were surface stained with 11 µL fluorescence-labeled monoclonal antibody cocktail (Table 2.8) for 15 min at 37 °C and in the dark in FACS tubes. After 5 min of incubation 1 µL L/D Red was supplemented to each sample. Next, staining was stopped by washing PBMCs with 4 mL PBS/BSA and centrifugation (10 min, 490 g, 4 °C). Then, stained cells were fixed by re-suspending them in 1 mL 1X FoxP3 fixation buffer. After an incubation of 30 min in the dark at 4 °C, fixed cells were washed once with PBS/BSA and spun down (10 min, 490 g, 4 °C). In order to permeabilize the samples, PBS/BSA supernatant was removed and cell pellets re-suspended in 1X FoxP3 permeabilization buffer. Cells were directly centrifuged (10 min, 490 g, 4 °C) and supernatant carefully removed. Next, PBMCs were again re-suspended in 70 µL 1X FoxP3 permeabilization buffer and intracellularly stained 30 min at RT with 30 µL antibody cocktail listed in Table 2.8. After a final washing step with 1X FoxP3 permeabilization buffer, cells were centrifuged (10 min, 490 g, 4 °C) and pellets re-suspended in 250 µL PBS/BSA. Samples were acquired on a LSR II flow cytometer with an optical filter configuration listed in table 2.1. An exemplary gating is shown in fig. 2.6. All calculations of absolute whole blood cell counts for this panel based on the $CD3^+$ gate.

Table 2.8: Antibody cocktails for T-cell activation panel

Name	Fluorochrome	Clone	Isotype	Vendor	Dilution
T-cell activation panel (LSR II) – surface staining					
CD8	BV570	RPA-T8	mouse IgG1	Biolegend	1:50
CD45RA	efluor605	HI100	mouse IgG2b	Ebioscience	1:100
CCR7	PerCpCy5.5	G043H7	mouse IgG2a	Biolegend	1:50
γδTCR	PE	11F2	mouse IgG1	BD Biosciences	1:20
CD19	PE-CF594	HIB19	mouse IgG1	BD Biosciences	1:100
CD14	PE-CF594	MϕP9	mouse IgG2b	BD Biosciences	1:100
CD38	PECy7	HIT2	mouse IgG1	Biolegend	1:100
CD4	Alexa700	OKT4	mouse IgG2b	Biolegend	1:200
HLA-DR	APCH7	G46-6	mouse IgG2a	BD Biosciences	1:100
Beriglobin	n.a.	n.a.	n.a.	Aventis	1:50
T-cell activation panel (LSR II) – intracellular staining					
Ki-67	V450	B56	mouse IgG1	BD Biosciences	1:50
CD3	V500	SP34-2	mouse IgG1	BD Biosciences	1:50
FoxP3	Alexa488	259D/C7	mouse IgG1	BD Biosciences	1:5
Helios	Alexa647	22F6	ar. hamster IgG	BD Biosciences	1:40
Beriglobin	n.a.	n.a.	n.a.	Aventis	1:50



conventional CD4$^+$ T cells by co-expression of the transcription factors FoxP3 and Helios. From all T-cell subsets we could determine the sub-phenotypes (Naive, CM, EM and Eff) by combinatorial expression of CD45RA and CCR7. Additionally, we could determine the activation/proliferation status by assessing CD38, HLA-DR and Ki-67. Although every combination of these markers is theoretically applicable, we choose for CD4$^+$ T cells co-expression of Ki-67/CD38 and for CD8$^+$ T cells CD38/HLA-DR for the definition of activated cells. Depicted is donor 15 (young) at day 2.

2.2.9.7 Assessment of specific CD4$^+$ T cells after YF vaccine stimulation

YF-specific CD4$^+$ T cells were measured after *ex vivo* stimulation of whole blood with vaccine at study days 0, 2, 4, 7, 10, 14, 17, 21 and 28. At each day and for each donor three stimulations were performed: negative control (un-stimulated), positive control (SEB/TSST1 stimulated) and a YF-vaccine stimulation. For each stimulation, 1 mL of freshly drawn heparinized blood was transferred into 12 mL round bottom stimulation tubes. Then stimulation cocktails were added as listed in table 2.9. Tubes were then capped permeable to air and incubated for 2 h at 37 °C under humid conditions and in 5 % CO_2 atmosphere. During the stimulation, tubes were locked in a 45° position. After 2 h, stimulation was shortly interrupted in order to supplement Brefeldin A (10 µg/mL) to each tube, preventing retrograde protein transport from the Golgi apparatus and thereby enabling intracellular detection of induced cytokine expression. After continuing the stimulation for another 4 h at same conditions, 100 µL 20 nM EDTA was added to each sample to stop the stimulation. Next, erythrocytes were lysed through addition of 10 mL Qiagen Buffer EL to each stimulation tube and intensive vortexing. Lysis reaction was increased by a 10 min incubation step on ice, during which tubes were repeatedly inverted. This was followed by a centrifugation step

(10 min, 490 g, 4 °C). Supernatant was carefully aspirated, including the viscous, orange erythrocyte debris resulting in whitish cell pellets which were washed once again with 4 mL Qiagen Buffer EL and centrifuged (10 min, 490 g, 4 °C). Next, cell pellets were re-suspended in PBS/BSA, transferred into 5 mL FACS tubes and washed with PBS/BSA (10 min, 490 g, 4 °C). Then, the protocol proceeded as outlined for the B-cell panel (surface staining, fixation, permeabilization and intracellular staining). The antibody cocktails used for both staining steps are listed in table 2.10. As live/dead discriminator 1 µL LD Red was used. Samples were measured on a LSR II flow cytometer with an optical filter setting shown in table 2.1. The gating strategy is depicted in fig. 2.7. All measured frequencies of cytokine or activation marker expressing cells were background subtracted by the matching un-stimulated sample. Calculation of whole blood counts based on the $CD4^+$ gate. For multi-dimensional assessment of cytokine expression a SPICE analysis was performed. For this a combinatorial Boolean gating of cytokine expression on vaccine-stimulated cells was conducted in FlowJo. The obtained relative frequencies of cytokine producing cells (percentages within $CD40L^+$ $CD4^+$ T cells) were imported into SPICE (Roederer *et al.*, 2011) and analyzed according to discriminators such as age, gender and/or study day. Polyfunctionality (number of co-expressed cytokines) was automatically assigned by SPICE and relative frequencies of single, double, triple, etc. cytokine producing cells was analyzed in a similar way in SPICE. Assessment of vaccine-specific $CD4^+$ T cells at the very late follow-up study day was conducted in a similar way with a modified FACS panel indicated in table 2.10.

Table 2.9: Stimulations used in the whole blood $CD4^+$ T cell stimulation panel

Stimulation	RPMI/AB	anti hu CD28	Stimulants	Distributed volume
Master mixes for whole blood $CD4^+$ T-cell stimulation				
SEB/TSST1	96.5 µL	1 µL	1.5 µL SEB 1.0 µL TSST1	100 µL
YF-vaccine	49.0 µL	1 µL	50 µL YF-vaccine (⅓ single dose)	100 µL
un-stimulated	99.0 µL	1 µL	-	100 µL

Table 2.10: Antibody cocktails for whole blood CD4+ T cell stimulation panel (acute phase and follow up)

Name	Fluorochrome	Clone	Isotype	Vendor	Dilution
Whole blood $CD4^+$ T-cell stimulation panel (LSR II) – surface staining					
CD8	V500	RPA-T8	mouse IgG1	BD Biosciences	1:50
CD19	PE-CF594	HIB19	mouse IgG1	BD Biosciences	1:100
CD14	PE-CF594	MϕP9	mouse IgG2b	BD Biosciences	1:100
CD4	A647	TT1	mouse IgG1	in house DRFZ	1:300
Beriglobin	n.a.	n.a.	n.a.	Aventis	1:50
Whole blood $CD4^+$ T-cell stimulation panel (LSR II) – intracellular staining					
CD40L	PacBlue	24-31	mouse IgG1	Biolegend	1:100
IL2	BV605	MQ1-17H12	rat IgG2a	Biolegend	1:200
CD3	efluor650	OKT3	mouse IgG2a	Ebioscience	1:50
IL4	Alexa488	MP4-25D2	rat IgG1	Biolegend	1:100
TNFα	PerCpCy5.5	MAb11	mouse IgG1	Biolegend	1:30
RANKL	PE	MIH24	mouse IgG2b	Biolegend	1:200
IL22	PECy7	22URTI	mouse IgG1	Ebioscience	1:200
IFNγ	Alexa700	B27	mouse IgG1	Biolegend	1:400
IL17	APCCy7	BL168	mouse IgG1	Biolegend	1:50
Beriglobin	n.a.	n.a.	n.a.	Aventis	1:50
Follow up day – Whole blood $CD4^+$ T-cell stimulation panel (LSR II) – surface staining					
CD8	BV510	RPA-T8	mouse IgG1	Biolegend	1:200
CCR7	PE	G043H7	mouse IgG2a	Biolegend	1:20
CD19	PE-CF594	HIB19	mouse IgG1	BD Biosciences	1:100
CD14	PE-CF594	MϕP9	mouse IgG2b	BD Biosciences	1:100
CD45RA	PECy7	L48	mouse IgG1	BD Biosciences	1:50
CD4	Cy5	TT1	mouse IgG1	in house DRFZ	1:600
Beriglobin	n.a.	n.a.	n.a.	Aventis	1:50
Follow up day – Whole blood $CD4^+$ T-cell stimulation panel (LSR II) – intracellular staining					
CD40L	BV421	24-31	mouse IgG1	Biolegend	1:200
IL2	BV605	MQ1-17H12	rat IgG2a	Biolegend	1:200
CD3	efluor650	OKT3	mouse IgG2a	Ebioscience	1:50
IL4	Alexa488	MP4-25D2	rat IgG1	Biolegend	1:100
TNFα	PerCpCy5.5	MAb11	mouse IgG1	Biolegend	1:30
IFNγ	Alexa700	B27	mouse IgG1	Biolegend	1:400
IL17	APCCy7	BL168	mouse IgG1	Biolegend	1:50
Beriglobin	n.a.	n.a.	n.a.	Aventis	1:50

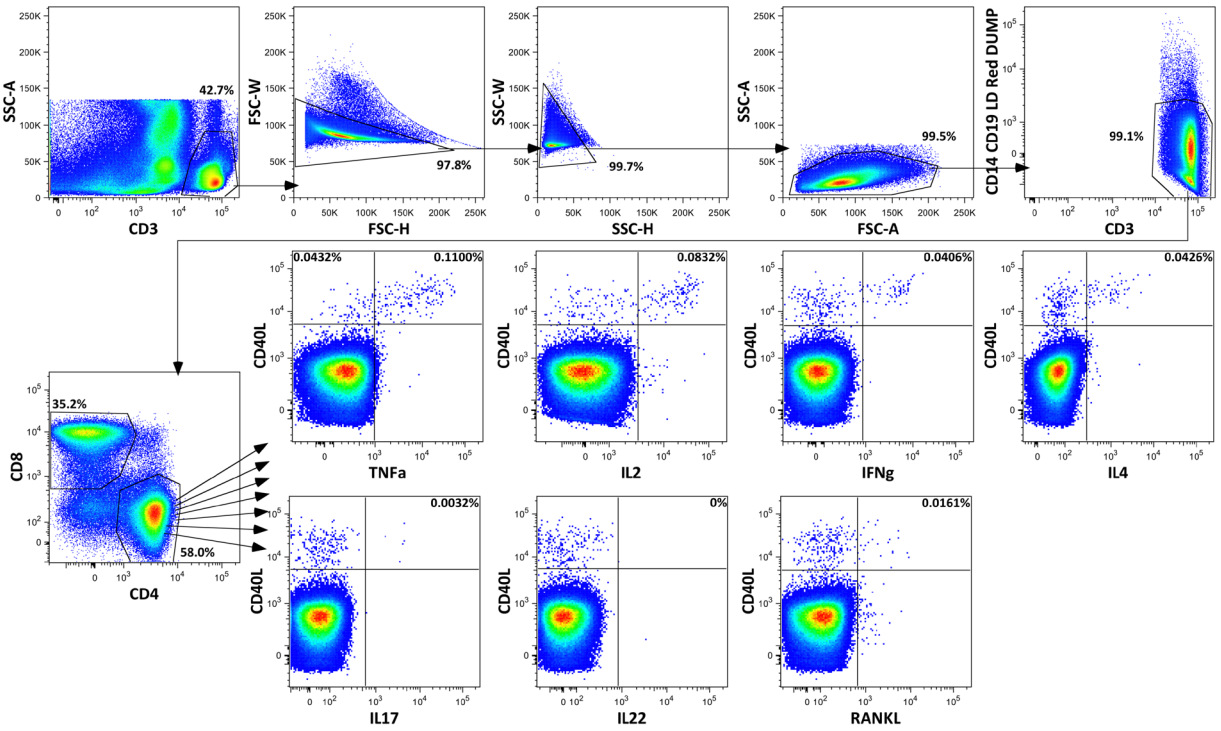


Figure 2.7: Gating strategy for the whole blood CD4$^+$ T-cell stimulation panel.
Activated and cytokine expressing CD4$^+$ T cells were assessed after antigen-specific stimulation in lysed and fixated whole blood by gating first on CD3$^+$ T cells. Next, cell aggregates were removed and gating was refined on cells with lymphocyte properties, excluding monocytes, B cells and dead cells. After distinguishing CD8$^+$ from CD4$^+$ T cells, we analyzed cytokine and activation marker (CD40L, RANKL) expression in CD4$^+$ T cells. If not otherwise mentioned co-expression of CD40L and a cytokine are reported in the results. For assessment of the qualitative CD4$^+$ T-cell response CD40L$^+$/TNFα$^{+/-}$ CD4$^+$ T cells were further used in a Boolean gating approach generating results for all possible cytokine combinations (not shown). Depicted is donor 15 (young) at day 10 after stimulation with YF vaccine.

2.2.9.8 Examination of YF-specific CD8$^+$ T cells and CMV/EBV-specific cellular responses

YF-specific CD8$^+$ T cells were measured after *ex vivo* stimulation of PBMCs in HLA-A0201 positive donors (n=10) at study days 0, 4, 7, 10, 14, 17, 21 and 28. In a similar fashion CMV and EBV-specific CD4$^+$ and CD8$^+$ T cells were studied in all donors at day 0. At each studied day and for each HLA-A0201$^+$ donor the following stimulations were performed: negative control (un-stimulated), positive control (SEB/TSST1 stimulated) and a NS4b 9mer peptide stimulation. It has been previously shown that for HLA-A0201$^+$ YF-vaccinees about 50 % of the virus-specific CD8$^+$ T-cell response is directed against this particular immunodominant epitope 214–222 of the NS4b protein (Akondy *et al.*, 2009). For the CMV and EBV analysis, the same positive and negative controls were used. CMV-specific T cells were assessed by stimulating PBMCs with pp65 and IE-1 15mer peptide pools. EBV-specific T cells were studied by stimulating PBMCs with EBNA1 and BZLF1 15mer peptide pools. For each stimulation, 5x10^6 PBMCs were transferred into 12 mL round bottom stimulation tubes in 500 µL RPMI/AB. Then, stimulation cocktails were added as listed in table 2.11, including Monensin A and anti-

CD107a antibodies enabling examination of cytotoxic functionality. The total stimulation volume was 1 mL. Tubes were then capped permeable to air and incubated for 2 h at 37 °C under humid conditions and in 5 % CO_2 atmosphere. During the stimulation, tubes were locked in a 45° position. After 2 h, stimulation was shortly interrupted in order to supplement Brefeldin A (10 µg/mL) to each tube. After continuing the stimulation for another 4 h at same conditions, 100 µL 20 nM EDTA was added to each sample to stop the stimulation. Then, stimulated PBMCs were washed once with 10 mL PBS/BSA (10 min, 490 g, 4 °C) and transferred into FACS tubes. The subsequent fixation, permeabilization and staining procedure followed the B-cell panel protocol (surface staining, fixation, permeabilization and intracellular staining). The antibody cocktails used for both staining steps are listed in table 2.12. As live/dead discriminator 1 µL LD Red was used. Samples were measured on a LSR II flow cytometer with an optical filter setting shown in table 2.1. The gating strategy is depicted in fig. 2.8. All measured frequencies of cytokine or activation marker expressing cells were background subtracted by the matching un-stimulated sample. Calculation of whole blood counts based on the $CD8^+$ gate. Assessment of YF-specific $CD8^+$ T cells at the very late follow-up study day was conducted in a similar way with a modified FACS panel indicated in table 2.12.

Table 2.11: Stimulations used for the assessment of YF-, CMV- or EBV-specific $CD8^+$ T cells

Stimulation	RPMI/AB	anti hu CD28	Monensin	anti hu CD107a A488	Stimulants	Distributed volume
Master mixes for PBMC stimulation (for assessment of YF-specific $CD8^+$ T cells)						
SEB/TSST1	495 µL	1 µL	0.7 µL	1.43 µL	1.5 µL SEB 1.0 µL TSST1	500 µL
NS4b 9mer	493 µL	1 µL	0.7 µL	1.43 µL	4.0 µL NS4b 9mer	500 µL
un-stimulated	493 µL	1 µL	0.7 µL	1.43 µL	4.0 µL DMSO/PBS	500 µL
Master mix for PBMC stimulation (for assessment of CMV/EBV-specific T cells at day 0)						
SEB/TSST1	495 µL	1 µL	0.7 µL	1.43 µL	1.5 µL SEB 1.0 µL TSST1	500 µL
CMV	489 µL	1 µL	0.7 µL	1.43 µL	4.0 µL pp65 pool 4.0 µL IE-1 pool	500 µL
EBV	489 µL	1 µL	0.7 µL	1.43 µL	4.0 µL EBNA1 pool 4.0 µL BZLF1 pool	500 µL
un-stimulated	489 µL	1 µL	0.7 µL	1.43 µL	8.0 µL DMSO/PBS	500 µL

Table 2.12: Antibody cocktails for PBMC stimulation panel (acute phase and follow up)

Beriglobin	n.a.	n.a.	n.a.	Aventis	1:50
PBMC stimulation panel (LSR II) – surface staining					
CD4	V500	RPA-T4	mouse IgG1	BD Biosciences	1:50
CD8	efluor605	RPA-T8	mouse IgG1	Ebioscience	1:50
Beriglobin	n.a.	n.a.	n.a.	Aventis	1:50
PBMC stimulation panel (LSR II) – intracellular staining					
CD40L	PacBlue	24-31	mouse IgG1	Biolegend	1:100
CD3	efluor650	OKT3	mouse IgG2a	Ebioscience	1:50
CD107a	Alexa488	H4A3	mouse IgG1	Biolegend	1:700 [1]
Granzyme A	PerCpCy5.5	CB9	mouse IgG1	Biolegend	1:20
Perforin	PE	B-D48	mouse IgG1	Abcam	1:50 [2]
IFNγ	PECy7	B27	mouse IgG1	Biolegend	1:100
IL2	APC	MQ1-17H12	rat IgG2a	Biolegend	1:200
Granzyme B	Alexa700	GB11	mouse IgG1	BD Bioscience	1:100
IL17	APCCy7	BL168	mouse IgG1	Biolegend	1:50
Beriglobin	n.a.	n.a.	n.a.	Aventis	1:50
Follow up day – PBMC stimulation panel (LSR II) – surface staining					
CD4	BV510	RPA-T4	mouse IgG1	Biolegend	1:100
CD45RA	BV605	HI100	mouse IgG2b	Biolegend	1:200
CD8	BV785	RPA-T8	mouse IgG1	Biolegend	1:200
CCR7	PerCpCy5.5	G043H7	mouse IgG2a	Biolegend	1:40
CD19	PE-CF594	HIB19	mouse IgG1	BD Biosciences	1:100
CD14	PE-CF594	MϕP9	mouse IgG2b	BD Biosciences	1:100
Beriglobin	n.a.	n.a.	n.a.	Aventis	1:50
Follow up day – PBMC stimulation panel (LSR II) – intracellular staining					
CD40L	BV421	24-31	mouse IgG1	Biolegend	1:200
CD3	BV650	OKT3	mouse IgG2a	Biolegend	1:50
CD107a	Alexa488	H4A3	mouse IgG1	Biolegend	1:700
Perforin	PE	B-D48	mouse IgG1	Abcam	1:50 [2]
IFNγ	PECy7	B27	mouse IgG1	Biolegend	1:100
IL2	APC	MQ1-17H12	rat IgG2a	Biolegend	1:200
Granzyme B	Alexa700	GB11	mouse IgG1	BD Bioscience	1:100
IL17	APCCy7	BL168	mouse IgG1	Biolegend	1:50
Beriglobin	n.a.	n.a.	n.a.	Aventis	1:50

[1] CD107a was added into the stimulation master mix

[2] Each lyophilized Perforin PE vial was solved in 500µL PBS prior usage

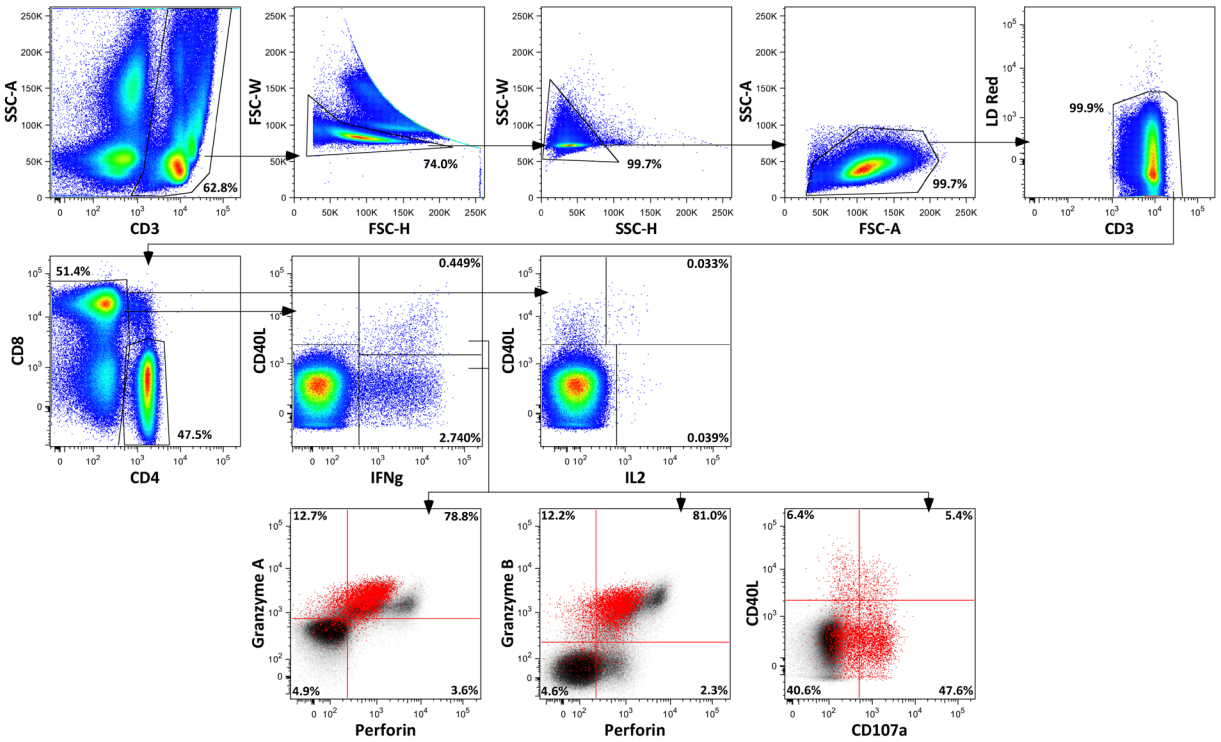


Figure 2.8: Gating strategy for the assessment of YF-specific CD8$^+$ T cells.
Activated and cytokine expressing CD8$^+$ T cells were assessed after antigen-specific stimulation in fixated PBMCs by gating first on CD3$^+$ T cells. Next, cell aggregates were removed and gating was refined on cells with lymphocyte properties, excluding dead cells. CD8$^+$ T cells were distinguished from CD4$^+$ T cells. The CD8 gate was drawn very large, as we noticed a strong down regulation of CD8 after stimulation with NS4b 9mer. In CD8$^+$ T cells we investigated expression of IFNγ, IL2 and CD40L. We further characterized the expression of markers involved in cytotoxicity, such as Perforin, Granzyme A, Granzyme B and CD107a in IFNγ$^+$ CD8$^+$ T cells which are depicted as an overlay (red) on total CD8$^+$ T cells. Assessment of activated and cytokine expressing CD4$^+$ T cells by this panel was analog to the whole blood CD4$^+$ stimulation panel (not shown). Depicted is donor 15 (young) at day 17 after stimulation with YF NS4b 9mer

2.2.10 Data acquisition, analysis and statistics

FACS data was acquired on LSR II by FACSDiva 6 and on MACSQuant by MACSQuantify Software. All FACS data were properly compensated using matching single-stain controls acquired 2-3 days prior beginning of each study round. Instrument stability was monitored over the study course using BD Rainbow Calibration Particles. Data was exported into fcs-data files and analyzed with Flowjo 9.7.4 (MacOS Version). Finalized, gated data was exported into data tables. For statistical analysis of any observed data GraphPad Prism 5 was used. Multivariate analysis of tabular data was conducted with R 3.02 (R Development Core Team, 2014) by Karsten Jürchott, AG Neumann. Z-scores for each marker were calculated by subtracting the mean and dividing by the standard deviation. Obtained values were used in a principle component analysis utilizing a singular value decomposition. The first two principle components were displayed in a scatter plot. Furthermore, a hierarchical clustering of the z-scores was performed using Euclidean distances and complete linkage. Z-scores of response

markers were calculated and color coded by applying the color gradient equidistantly from minimum to maximum of each marker separately. In regard to statistics, this study had an exploratory character with no initial assumptions regarding the effect sizes. Since a power test was therefore not possible, the size of the cohort was limited by available donors during the study time. The study was performed in 3 independent rounds (n=5, n=5 and n=13). In order to avoid an inappropriate increase of type II errors, no adjustment of the significance level for multiple testing was done. The Shapiro test was used to test normal distribution of the data, which was rejected for most of the groups ($p<0.05$). Unless stated otherwise, the two-sided Mann-Whitney-U test as a non-parametric test to compare differences in groups was used. Data were tested for homoscedasticity as a prerequisite for the Mann-Whitney-U test with the Levene's test ($p<0.05$). If not mentioned otherwise, Pearson's correlation was used to determine correlations. A p-value of ≤ 0.05 was required for significance in group and correlation analyses (actual p-values are listed in figure legends). Asterisks in figures indicate levels of significance (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

3. Results

3.1 Serum viremia

In order to explore age-related differences in virus clearance we first investigated serum viremia in both age groups. In collaboration with Prof. Dr. Niedrig's group at the RKI, we measured viremia by RT-PCR (Chap. 2.2.3) before (day 0) and at days 2, 4, 7 and 10 after vaccination with the live-attenuated YFV-17D strain. We detected in all except one of the elderly subjects (91.7 %) virus in the blood, whereas only 8 of 11 (73.7 %) young vaccinees had detectable viremia. In those vaccinees that developed serum viremia no age-related difference of the absolute amount of virus in serum was observed (data not shown). However, we noticed that maximal viral load occurred later in the elderly. Serum viremia peaked in two elderly subjects at day 7 and in two others even at day 10, which was not observed in any young vaccinee. Hence late viral burden was significantly associated with age (Fig. 3.1), which led us to hypothesize that anti-viral effector functions were affected in the elderly.

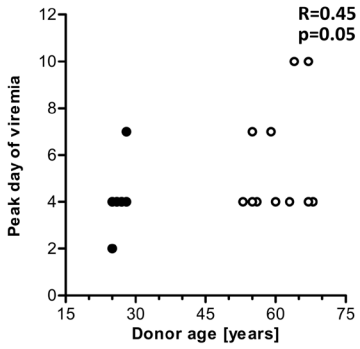


Figure 3.1: Maximal viral load and age
Individual peak days of viremia (day of maximum YF-genome equivalents per mL serum) versus donor's age (young: n=8, elderly: n=11). Individuals, who were tested negatively for viremia at all time-points (n=4/23) were not included in this analysis. Testing of correlation was performed by Spearman correlation. Young: filled circles; elderly: open circles

3.2 The acute humoral response

As one essential element of antiviral immune effector mechanisms we studied the humoral response before and at eight consecutive time points after vaccination. Prior to vaccination we assured by performing an anti-Flavivirus mosaic IIFA that all study participants were immunologically truly naive to YFV-17D, i.e. they did not have any detectable antibodies to YFV, TBE, JEV, WNV or DENV (Chap. 2.2.2). Next, three different immunoglobulin (Ig) classes directed against YFV-17D were assessed in a collaboration with the group of Prof. Dr. Niedrig in the sera of all vaccinees by an anti-YFV IIFA (Chap. 2.2.4). As shown in fig. 3.2A-C, we observed the profile of a typical primary immune response starting with the generation of YF-specific IgM antibodies around day 7 followed by the production of YF-specific IgG antibodies from day 10 onwards. We also measured YF-specific IgA antibodies that followed the same

kinetics as the specific IgG antibodies. By day 14 all vaccinees had developed YF-specific IgM, and by day 17 YF-specific IgG and IgA titers, indicating that all immunized subjects were responding to the vaccination. Limited by the semi-quantitative nature of the IIFA we could not detect any age-specific differences in antibody titers directed against YFV-17D. However, we observed the trend of a delayed onset of YF-specific IgM production in the elderly (Fig. 3.2D) prompting us to investigate YF-neutralizing antibodies by a quantitative PRNT90 performed at the RKI (Chap. 2.2.5). All vaccinees, regardless of age, developed protective YF-

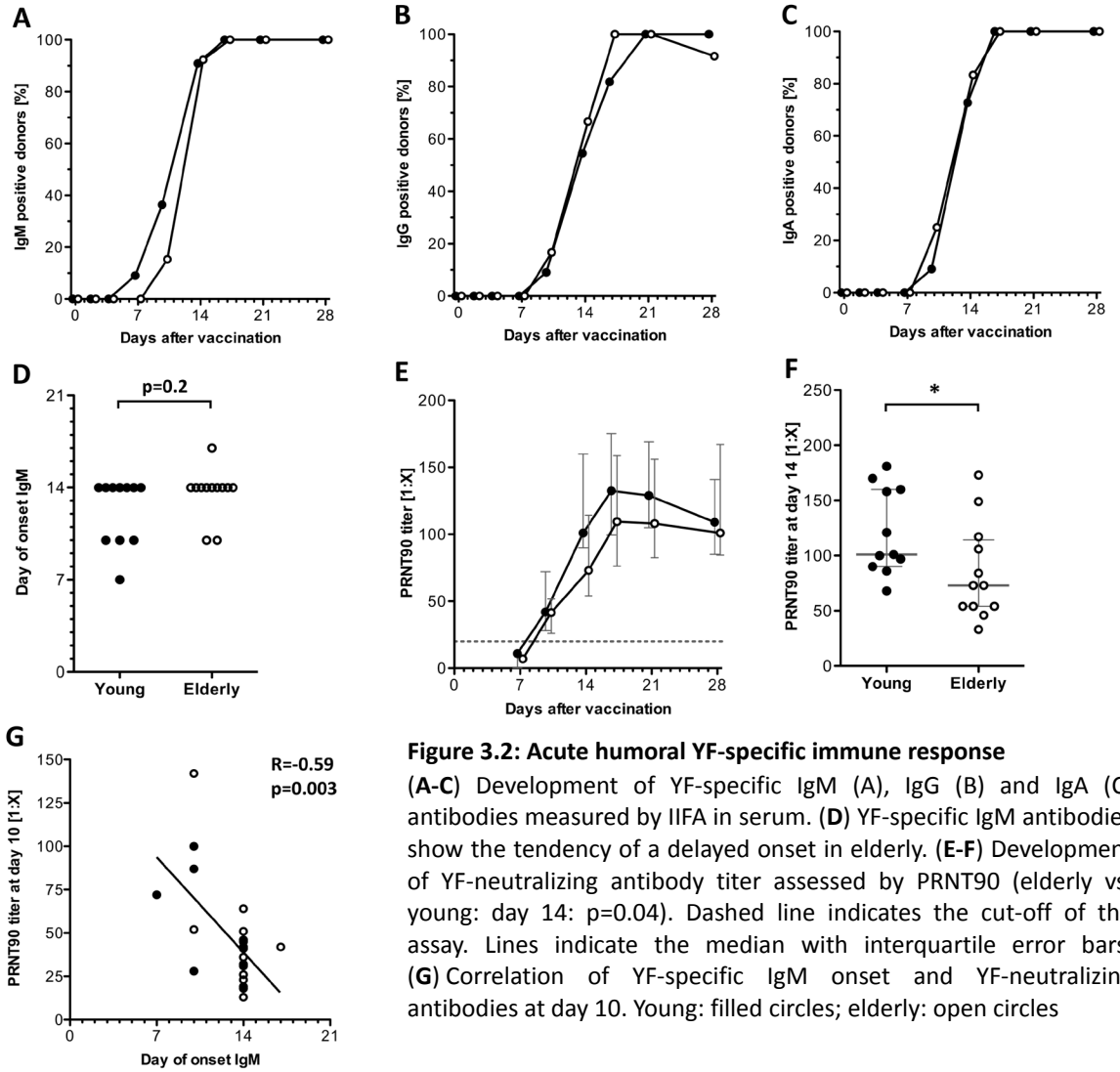


Figure 3.2: Acute humoral YF-specific immune response
(**A-C**) Development of YF-specific IgM (A), IgG (B) and IgA (C) antibodies measured by IIFA in serum. (**D**) YF-specific IgM antibodies show the tendency of a delayed onset in elderly. (**E-F**) Development of YF-neutralizing antibody titer assessed by PRNT90 (elderly vs. young: day 14: p=0.04). Dashed line indicates the cut-off of the assay. Lines indicate the median with interquartile error bars. (**G**) Correlation of YF-specific IgM onset and YF-neutralizing antibodies at day 10. Young: filled circles; elderly: open circles

neutralizing titers (>1:20) by day 10 (Fig. 3.2E). The peak of neutralizing antibodies was reached on day 17 in both age groups. Interestingly, neutralizing titers were distinctly lower in aged individuals on day 14 (Fig. 3.2F, p=0.03), a finding that tended to continue until day 21. By day 28 neutralizing titers were comparable between both age groups with titers around 1:100. In conclusion, our investigation of the YF-specific humoral response revealed an age-related lag in the generation of YF-neutralizing antibodies, which can most likely be explained

by a deferred onset of anti-viral IgM production in the elderly, as indicated by a correlation of IgM onset and neutralizing antibody titers at day 10 (Fig. 3.2G).

3.3 Plasmablasts and the B-cell compartment

After immunization specific antibodies are produced by antibody secreting cells (ASC) that transiently appear in blood and are mostly comprised of acutely generated plasmablasts deriving from the germinal center reaction (Fink, 2012). We monitored plasmablasts as $CD19^{low}/CD20^{neg}/CD27^{hi}/CD38^{hi}$ cells by FACS in the course of the YF immune response (Fig. 3.3A) (Chap. 2.2.9.4). Regardless of age, we detected a very distinct and transient peak of acute plasmablasts around day 14 after vaccination (Fig. 3.3B). Remarkably, the magnitude of

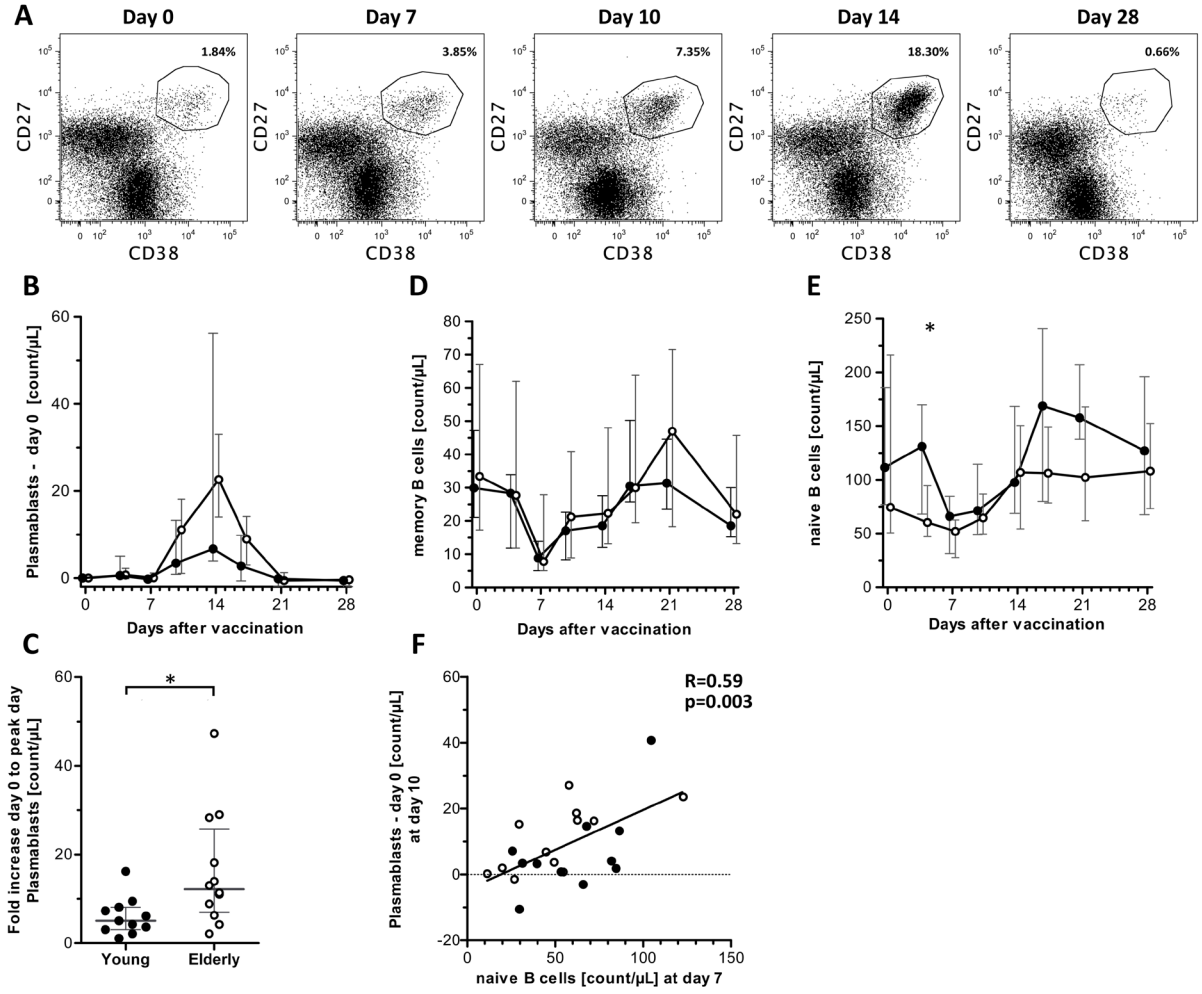


Figure 3.3: Acute B-cell response
(**A**) Example of acutely generated plasmablasts in elderly donor 25. Depicted cells are pre-gated on live, $CD19^{low/+}$ B cells. (**B**) Absolute, baseline-corrected counts of plasmablasts after YF-vaccination. (**C**) Increased fold change of plasmablasts from day 0 to individual peak days based on absolute counts in the elderly group (p=0.02). (**D-E**) Absolute counts of memory ($IgD^-/CD27^+$) and naive ($IgD^+/CD27^-$) B cells after YF vaccination (naive B cells: elderly vs. young: day 4: p=0.05). (**F**) Positive correlation of naive B cell counts at day 7 with baseline-corrected plasmablast counts at day 10. A similar finding could be obtained with plasmablast counts of day 14. Lines indicate the median with interquartile error bars. Young: filled circles; elderly: open circles

the plasmablast peak (as fold increase to baseline) was significant higher in elderly individuals (Fig. 3.3C, p=0.02), however, this did not positively correlate with neutralizing antibody titers (data not shown).

By the same FACS panel we also analyzed naive ($CD27^-/IgD^+$) and memory ($CD27^+/IgD^-$) $CD19^+$ B cells in the course of the response. Both B-cell subsets showed distinct kinetics characterized by a highly significant drop in cell numbers from day 0 to day 7. After day 7 absolute counts of both subsets increased again, peaking around days 14/17 for naive B cells and at day 21 for memory B cells (Fig. 3.3, D and E). Age differences were found only at day 4 in naive B cells.

By correlation analysis we wanted to assess the influence of the size of the naive B-cell compartment on the subsequent response. The naive B-cell status prior vaccination (day 0) revealed no significant correlation to neutralizing antibody titers or plasmablast counts (not shown). However, we observed a very significant positive correlation between numbers of naive B cells at day 7 and plasmablast at day 10 and 14, i.e. vaccinees, with comparably high numbers at the naive B-cell minimum generated subsequently more plasmablasts (Fig. 3.3F). Altogether we could observe a distinct response pattern in the B-cell compartment to YF vaccination that includes an exaggerated plasmablast response in the elderly.

3.4 The YF-induced, acute $CD8^+$ T-cell response

Cytotoxic $CD8^+$ T cells (CTLs) capable of eliminating virus-infected cells represent the other essential effector element in immune protection. Hence, we monitored YF-specific $CD8^+$ T cells over the course of the immune response, using the activation markers CD38 and HLA-DR (Fig. 3.4A) (Chap. 2.2.9.6). Co-expression of both markers can be used to track YF-specific activated $CD8^+$ T cells after vaccination (Ahmed and Akondy, 2011). We detected a strong increase of activated $HLA\text{-}DR^+/CD38^+$ $CD8^+$ T cells at day 14 and 17, which returned to basal levels by day 28 (Fig. 3.4B). Importantly, older vaccinees showed significantly (p=0.04, day 10; p=0.02, day 14) weaker CTL responses.

We alternatively assessed YF-specific $CD8^+$ T cells by intracellular cytokine staining (ICS) after stimulation with a YF-derived peptide (Chap. 2.2.9.8). As this peptide is HLA-A0201 restricted we could only analyze vaccinees possessing this particular HLA type ($HLA\text{-}A2^+$: n=10, young: n=5, elderly n=5). YF-peptide specific, IFNγ producing $CD8^+$ T cells could be detected from day 7 onwards (Fig. 3.4C). The strongest increase was observed from day 10 to day 14,

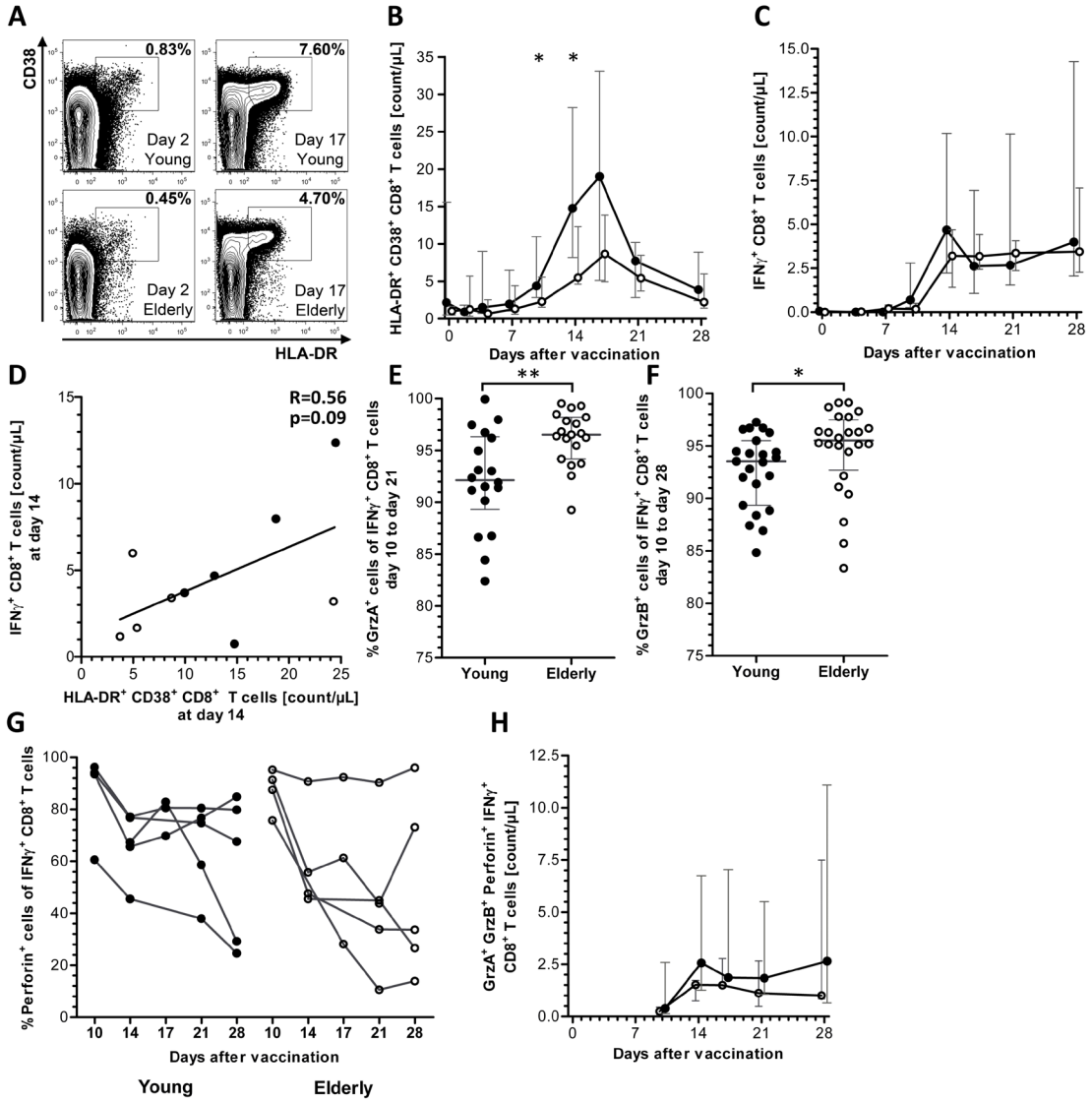


Figure 3.4: Acute CD8$^+$ T cell response

(**A**) Examples of acutely activated HLA-DR$^+$/CD38$^+$ CD8+ T cells in a young and old donor very early at day 2 after vaccination and at the peak at day 17. CD8$^+$ T cells were gated on live, CD3$^+$ cells. (**B**) Absolute counts of acutely activated HLA-DR$^+$/CD38$^+$ CD8$^+$ T cells after YF-vaccination (elderly vs. young: day10: p=0.04; day 14: p=0.02). (**C**) Absolute counts of IFNγ$^+$ CD8$^+$ T cells after re-stimulation with NS4b 9mer after YF-vaccination of HLA-A0201 subjects (n=10). (**D**) Positive correlation between counts of HLA DR$^+$/CD38$^+$ and IFNγ$^+$ CD8+ T cells at day 14 post vaccination. (**E-F**) Percentages of Granzyme A (E) or Granzyme B (F) expression within YF-specific IFNγ$^+$ CD8$^+$ T cells. Results of 10 vaccinees from day 10 to day 28 are stratified by age. Granzyme A: p=0.005; Granzyme B: p=0.03 (**G**) Percentage of Perforin expressing cells among YF-specific IFNγ$^+$ CD8$^+$ T cells. Individual data points are connected by gray lines. (**H**) Absolute counts of Perforin$^+$/Granzyme A$^+$/Granzyme B$^+$/IFNγ$^+$ CD8$^+$ T cells after YF-vaccination of HLA-A0201 subjects (n=10). Lines indicate the median with interquartile error bars. Young: filled circles; elderly: open circles

reaching a plateau phase lasting at least until day 28. Due to the low number of analyzed vaccinees no significant age-related differences in cell numbers could be detected, however we were able to correlate ICS data with the CD38/HLA-DR results. As shown in fig. 3.4D numbers of activated HLA-DR$^+$/CD38$^+$ CD8$^+$ T cells at day 14 correlated almost linearly with YF-specific IFNγ$^+$ CD8$^+$ T cells at day 14 (with the exception of 3 subjects). Assessing HLA-DR/CD38 is hence an appropriate surrogate marker combination for measuring the YF-specific CTL

response in the acute phase.

Moreover, we obtained in HLA-A0201$^+$ vaccinees qualitative functionality of YF-specific IFNγ$^+$ CD8$^+$ T cells from day 10 onwards. As illustrated in fig. 3.4, E and F, about 90 % of these cells expressed GrzA and B at all investigated days with significantly higher percentages in the elderly cohort. In contrast, relative frequencies of Perforin$^+$ cells decreased over time and were rather divers among donors but by trend lower in the elderly (Fig. 3.4G). Back-calculating these results to absolute counts of YF-specific GrzA$^+$ GrzB$^+$ Perforin$^+$ IFNγ$^+$ CD8$^+$ T cells, there was a trend that numbers of these cells were lower in the elderly individuals (Fig. 3.4H). Altogether, our data suggested diminished acute YF-specific CD8$^+$ T-cell immunity in elderly vaccinees, characterized by lower absolute cell numbers and possibly constrains in Perforin-mediated cytotoxicity.

3.5 The YF-induced, acute CD4$^+$ T-cell response

As both humoral and cellular anti-viral defense mechanisms, which are largely orchestrated by CD4$^+$ T helper cells, seemed to be compromised in the elderly, we also studied the CD4$^+$ T-cell compartment after YF vaccination. As proposed by Blom, we tracked activated YF-specific CD4$^+$ T cells by the co-expression of CD38 and the proliferation marker Ki-67 (Fig. 3.5A) (Chap. 2.2.9.6) (Blom *et al.*, 2013). In both age groups, the number of activated CD4$^+$ T cells increased strongly until day 10 (Fig. 3.5B). Interestingly, in almost all young vaccinees, the highest number of activated and proliferating CD4$^+$ T cells was reached at day 10 (Fig. 3.5C). In contrast, in about 50 % of the elderly, CD4$^+$ T cells were still expanding until day 14. Thus, the overall expansion was significantly increased (p=0.006) in the elderly cohort (Fig. 3.5D).

We alternatively assessed YF-specific CD4$^+$ T cells also by ICS after stimulation with YF-vaccine (Chap. 2.2.9.7). Specific CD4$^+$ T cells were identified by the expression of CD40L and various cytokines. The response was dominated by type 1 T-helper cells (T_H1) expressing IL2, TNFα and IFNγ. However, smaller fractions of cells also expressed the type 2 T-helper (T_H2) cytokine IL4 or type 17 T-helper (T_H17) cytokines IL17 and IL22 (Fig. 2.7). T_H1 and T_H2 cytokine expressing T cells behaved kinetically very similar: Percentages and numbers of these cells in blood strongly increased from day 4 onwards, reached a peak at day 14 and then began to decrease till the end of the observation window (Day 28) (Fig. 3.6A-D, Suppl. fig. 1A-D). In contrast, T_H17/T_H22 cytokine producers showed a totally different kinetic profile: They

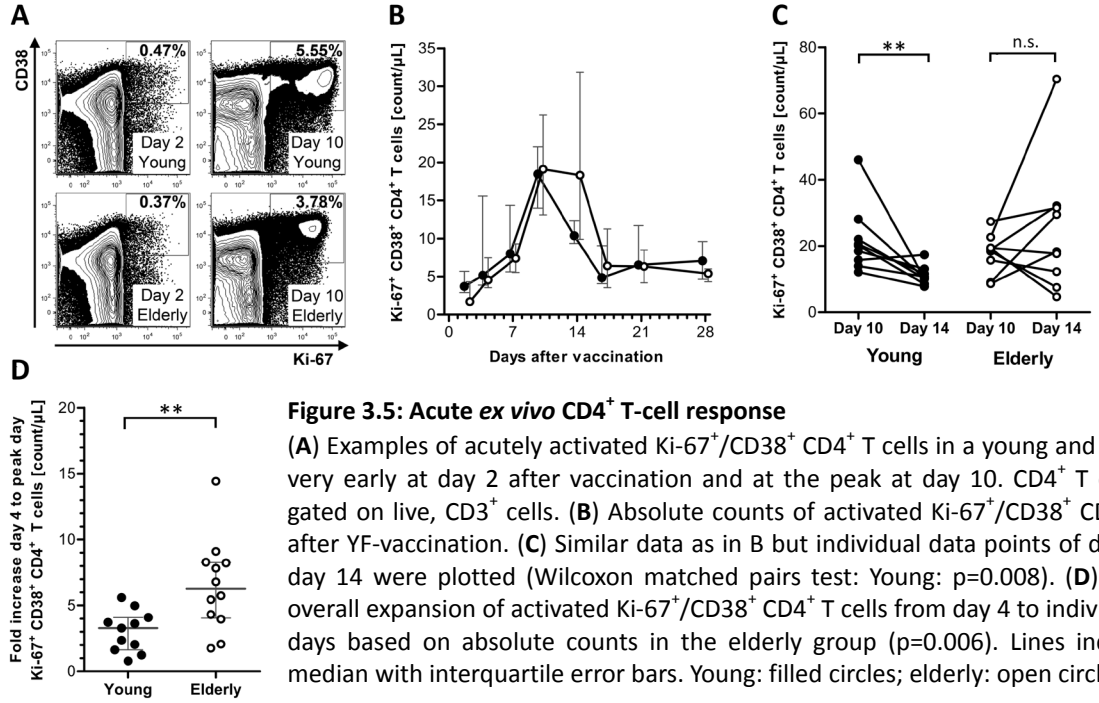


Figure 3.5: Acute *ex vivo* CD4$^+$ T-cell response

(**A**) Examples of acutely activated Ki-67$^+$/CD38$^+$ CD4$^+$ T cells in a young and old donor very early at day 2 after vaccination and at the peak at day 10. CD4$^+$ T cells were gated on live, CD3$^+$ cells. (**B**) Absolute counts of activated Ki-67$^+$/CD38$^+$ CD4$^+$ T cells after YF-vaccination. (**C**) Similar data as in B but individual data points of day 10 and day 14 were plotted (Wilcoxon matched pairs test: Young: p=0.008). (**D**) Increased overall expansion of activated Ki-67$^+$/CD38$^+$ CD4$^+$ T cells from day 4 to individual peak days based on absolute counts in the elderly group (p=0.006). Lines indicate the median with interquartile error bars. Young: filled circles; elderly: open circles

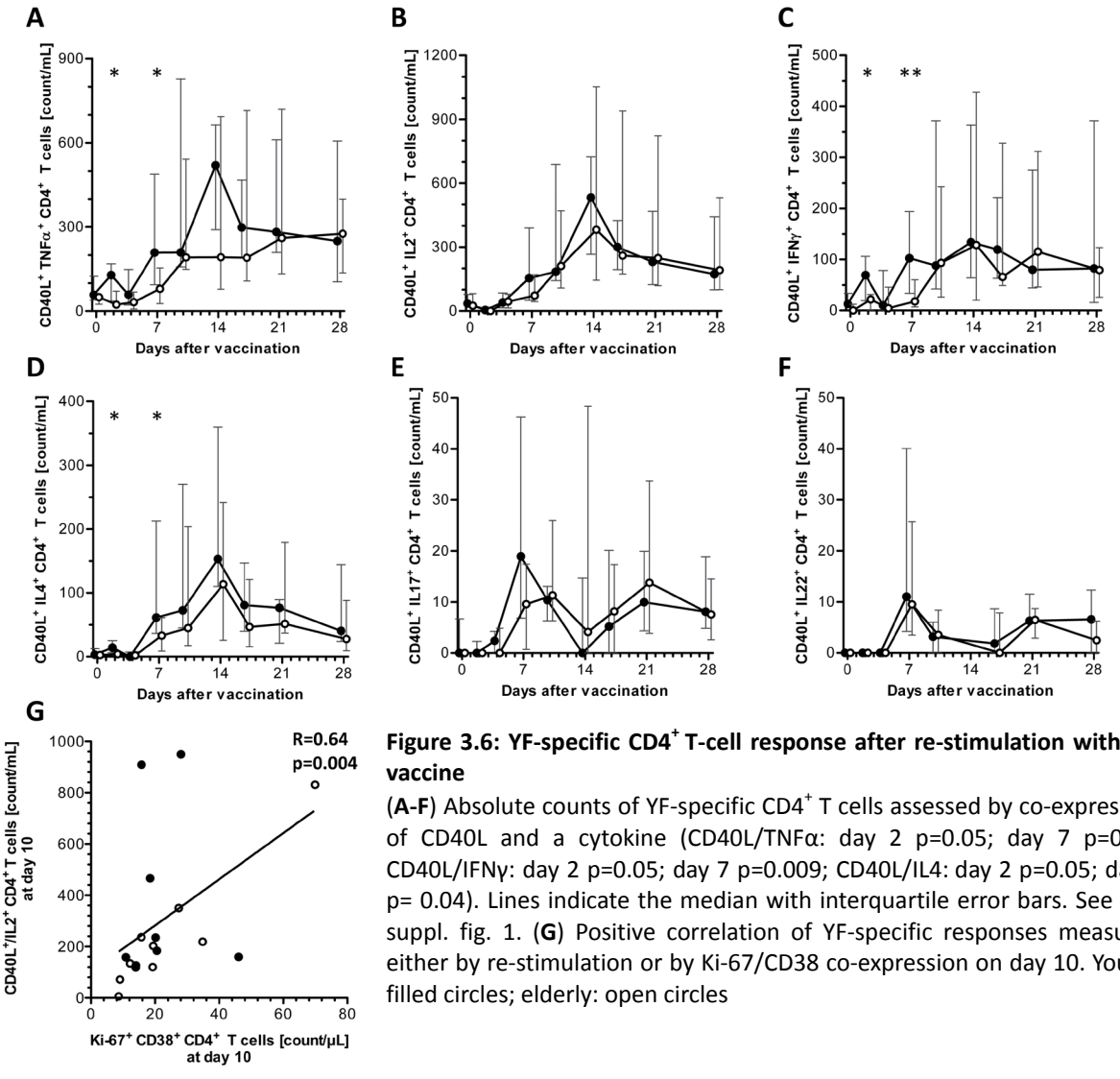


Figure 3.6: YF-specific CD4$^+$ T-cell response after re-stimulation with YF-vaccine

(**A-F**) Absolute counts of YF-specific CD4$^+$ T cells assessed by co-expression of CD40L and a cytokine (CD40L/TNFα: day 2 p=0.05; day 7 p=0.03; CD40L/IFNγ: day 2 p=0.05; day 7 p=0.009; CD40L/IL4: day 2 p=0.05; day 7 p= 0.04). Lines indicate the median with interquartile error bars. See also suppl. fig. 1. (**G**) Positive correlation of YF-specific responses measured either by re-stimulation or by Ki-67/CD38 co-expression on day 10. Young: filled circles; elderly: open circles

reached their maximum already at day 7, decreased towards day 14 and had a smaller second maximum at day 21 (Fig. 3.6, E and F; Suppl. fig. 1, E and F). Another interesting observation was the transient appearance of a small but significant number of TNFα, IFNγ or IL4 producing YF-specific $CD4^+$ T cells at day 2 that was more prominent in young individuals. Significantly higher numbers of TNFα, IFNγ or IL4 producers were also found in the expansion phase at day 7 in the young cohort. For validation, we compared the ICS results with the Ki-67/CD38 assessment of activated $CD4^+$ T cells and found a significant positive correlation e.g. at day 10 (Fig. 3.6G), indicating a good correspondence of both determination methods in the acute phase.

Next, we investigated the possible association of the $CD4^+$ T-cell response with anti-viral effector mechanisms. As shown in fig. 3.7, A and B for day 10, numbers of YF-specific Ki-67^+ $CD38^+$ $CD4^+$ T cells in the expansion phase (days 4 - 10) correlated positively with neutralizing antibody titers and numbers of YF-specific CTLs at day 14. In consequence, individuals with more YF-specific $CD4^+$ T cells in the expansion phase, e.g. at day 7, controlled virus earlier (Fig. 3.7C). In turn, vaccinees with an exaggerated and prolonged expansion of YF-specific

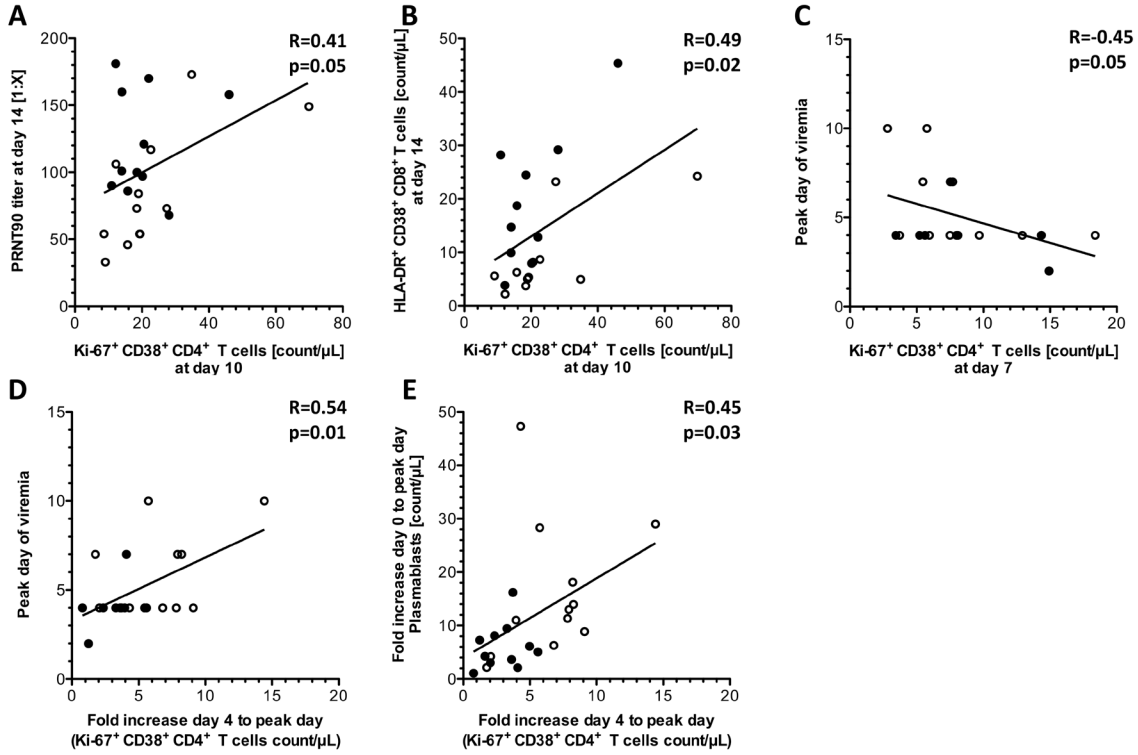


Figure 3.7: Correlation of quantitative, YF-specific $CD4^+$ T-cell response with other immunological read-outs and YF viremia

(**A, B**) Numbers of Ki-67^+/$CD38^+$ $CD4^+$ T cells at day 10 positively correlate with (A) YF-neutralizing antibody titers at day 14 and (B) with numbers of HLA-DR^+/$CD38^+$ $CD8^+$ T cells at day 14. (**C**) There is a negative correlation between numbers of Ki-67^+/$CD38^+$ $CD4^+$ T cells at day 7 and the peak days of viremia. Total expansion of specific $CD4^+$ T cells, measured as fold change day 4 to individual peak days, is associated (**D**) with late viral burden and (**E**) with the plasmablast peak (fold change). Young: filled circles; elderly: open circles

$CD4^+$ T cells, which was characteristic for the elderly (Fig. 3.5, C and D), experienced a delayed virus control (Fig. 3.7D). This was further supported by a significant correlation between YF-specific $CD4^+$ T-cell expansion and the magnitude of the plasmablast peak (Fig. 3.7E). Collectively, YF-specific $CD4^+$ T cells in the expansion phase (day 4 to day 10) positively affected anti-viral effector mechanisms and thereby promoted viral clearance. In contrast, vaccinees with a diminished virus control - mostly elderly - were more likely to experience an exaggerated $CD4^+$ T-cell and plasmablast response.

3.6 Functional analysis of YF-specific $CD4^+$ T cells

We next performed an in-depth analysis of the YF-specific $CD4^+$ T-cell compartment beyond the single cytokine level. For this we conducted a combinatorial SPICE analysis as proposed by Roeder *et al.* (Chap. 2.2.9.7) (Roederer *et al.*, 2011). Figure 3.8A depicts the composition within the YF-specific $CD40L^+$ $CD4^+$ T cells at day 14 and reveals that the response is dominated by some cytokine combinations, typically defined as T_H1 $CD4^+$ T cells (red arrows). Interestingly, IL4 producing subpopulations were mostly co-expressing classic T_H1 cytokines such as TNFα and/or IFNγ (green arrows), whereas IL4 single producers were rarely observed. As expected from the IL17 cytokine plots (Fig. 3.6E), subpopulations expressing IL17 were virtually absent at day 14 after vaccination. Next, we compared the $CD4^+$ T cell composition over time in and between the two age groups and performed a nonparametric permutation analysis of cytokine compositions acquired at day 7, day 14 and day 28 after vaccination (Fig. 3.8B). The test revealed significant compositional changes between day 7/14 and day 14/17 within each age group but no differences between age groups. A complete compositional time course of the 10 dominant YF-specific $CD4^+$ T-cell subpopulations is illustrated in figure 3.8C. The data indicated an increase of the relative frequency of IL2 single producers (subpop. G) and a decrease of TNFα single producers (subpop. D) towards day 14, which returned to initial levels at day 28. This observed change was significantly more prominent in the elderly and strongly associated with the Ki-67^+/$CD38^+$ $CD4^+$ T-cell expansion, increased in the aged cohort (Fig. 3.8D). Elderly had furthermore significantly lower relative frequencies of IL2/TNFα/IFNγ/IL4 quadruple (subpop. O) and TNFα/IFNγ double producers (subpop. F) at day 17 but more IFNγ single producers (subpop. C) at day 14 than young vaccinees.

This observation was further supported when we analyzed overall polyfunctionality that has been reported to be a crucial factor in defining protective T-cell responses (Seder *et al.*, 2008).

As shown in figure 3.9A, elderly vaccinees had significantly lower relative accumulated frequencies of quadruple and triple cytokine producers and instead higher frequencies of single cytokine producers in the YF-specific CD4$^+$ T-cell compartment.

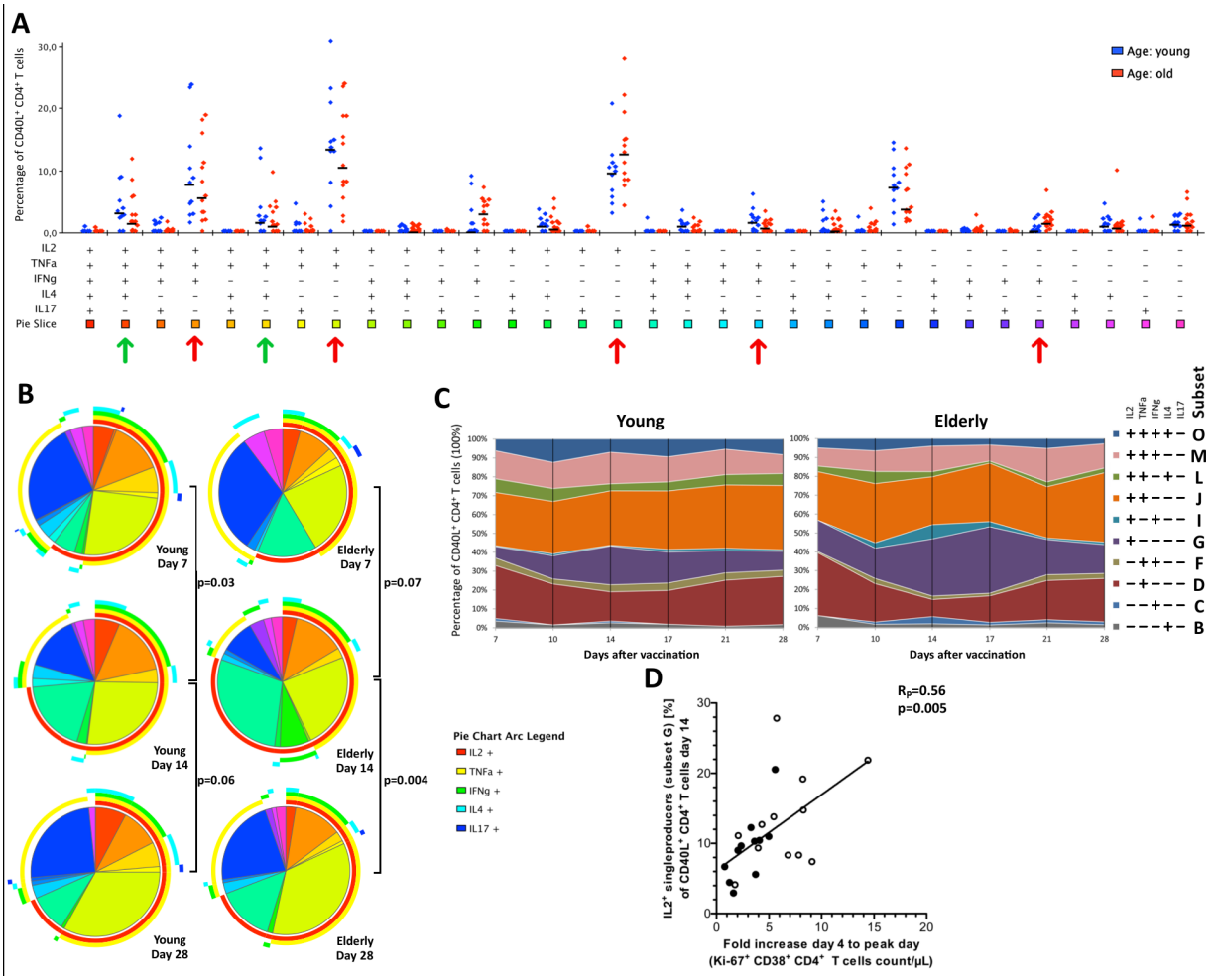


Figure 3.8: Qualitative composition of the YF-specific CD4$^+$ T-cell response
(**A**) Age comparison of qualitative YF-specific CD4$^+$ T-cell responses at day 14. Young: blue dots; Old: red dots. Black bars depict medians in each subset. Red arrows indicate typical T_H1 CD4$^+$ subsets, Green arrows indicate T_H1 CD4$^+$ subsets co-expressing IL4. (**B**) Pie charts based on median values for both age groups at day 7, 14 and 28. Pie slice color code is indicated in A. P-values of partial permutation tests (pie comparisons) are indicated. (**C**) Time course of the YF-specific, qualitative CD4$^+$ composition for selected 8 major subsets of young (left) and elderly (right) vaccinees. Charts base on median values of indicated subsets. Significant age differences were found in: Day 14: Subset C p=0.05; Day 17: Subset F p=0.02; Subset G p=0.03; Subset O p=0.03. (**D**) Significant correlation between total expansion of Ki-67$^+$ CD38$^+$ CD4$^+$ T cells and percentage of YF-specific IL2$^+$ single producers (subset G) at day 14. Young: filled circles; elderly: open circles

Altogether, the in-depth analysis of the YF-specific CD4$^+$ T-cell compartment revealed in addition to age-related quantitative also qualitative differences, such as a decreased level of polyfunctionality, which prompted us to investigate the impact on anti-viral effector mechanisms. Indeed, the relative percentage of YF-specific quadruple (and also quintuple) cytokine producers positively correlated with the YF-specific HLA-DR$^+$/CD38$^+$ CD8$^+$ T-cell response at day 14 (Fig. 3.9B). In turn, we observed a negative correlation between the

frequency of single cytokine producers and neutralizing antibodies at day 14 (Fig. 3.9C). Moreover, we found a very strong positive association between the degree of polyfunctionality and the quantity of the YF-specific cytokine response of $CD4^+$ T cells in the expansion phase (Fig. 3.9, D and E). In conclusion the quality of the YF-specific $CD4^+$ T-cell response is not only linked with the quantity of the $CD4^+$ T-cell response but also crucially influences subsequent anti-viral effector mechanisms.

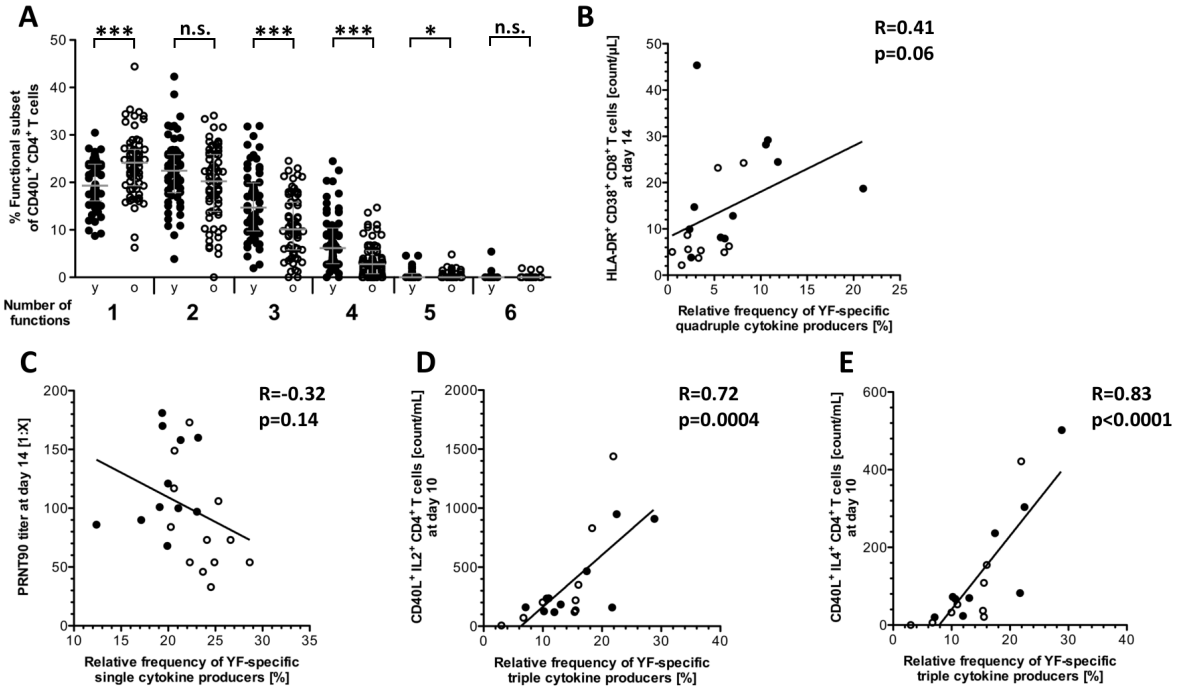


Figure 3.9: Polyfunctionality within YF-specific $CD4^+$ T cells

(**A**) Age comparison of single/polyfunctionality of YF-specific $CD40L^+$ $CD4^+$ T cells. Vaccine stimulation data from day 7 to day 28 are superimposed. Number of functions is calculated on expression of TNFα, IFNγ, IL2, IL4, IL17 and RANKL. (1 fct: p=0.0002; 3 fcts: p=0.0006; 4 fcts: p<0.0001; 5 fcts: p=0.02) (**B**) Correlation between percentage of quadruple functionality and numbers of activated HLA-DR^+/$CD38^+$ $CD8^+$ T cells at day 14 after vaccination. A similar correlation can be found for quintuple functionality. (**C**) Negative correlation between frequency of single functionality and YF-neutralizing titers at day 14 after vaccination. (**D, E**) Strong positive correlations between triple functionality and numbers of YF-specific CD40L/IL2 (D) or CD40L/IL4 (E) $CD4^+$ T cells at day 10 after vaccination. Similar correlations exist with quadruple and quintuple functionality. Young: filled circles; elderly: open circles.

3.7 Immunological signatures affecting subsequent anti-viral immunity

3.7.1 Influence of naive T cells and recent thymic emigrants (RTE)

As we had discovered age-specific alterations at several levels of the cellular and humoral anti-viral response we sought to identify immunological signatures manifested before or shortly after vaccination that critically govern the subsequent anti-viral mechanisms.

We hypothesized that the phenotypic composition of the T-cell compartment prior vaccination might influence the subsequent response. Hence, we assessed the expression of

CD45RA and CCR7 in CD8$^+$ T cells at day 0, which defines four subsets as proposed by Salusto *et al.*: Naive (N), central-memory (CM), effector-memory (EM) and effector (Eff) CD8$^+$ T cells (Sallusto *et al.*, 1999) (Chap. 2.2.9.3) (Fig. 2.3). Importantly, we observed highly significantly lower numbers and percentages of naive CD8$^+$ T cells in the elderly (Fig. 3.10A). In contrast, numbers of CM, EM and Eff CD8$^+$ T-cell subsets were independent of age in our study. We also investigated the phenotypic distribution in the CD4$^+$ T-cell compartment. There, we neither detected an age-difference in numbers of naive nor in CM, EM and Eff subsets (Fig. 3.10B). We next asked whether the reduced numbers of naive CD8$^+$ T cells affect the subsequent YF-specific CTL response. Indeed, we found a significant positive correlation between numbers of naive CD8$^+$ T cells prior vaccination and YF-specific HLA-DR$^+$/CD38$^+$ CD8$^+$ T cells at day 14 (Fig. 3.10C) indicating that a compromised naive CD8$^+$ T-cell compartment directly translates into a diminished CTL response in a primary infection.

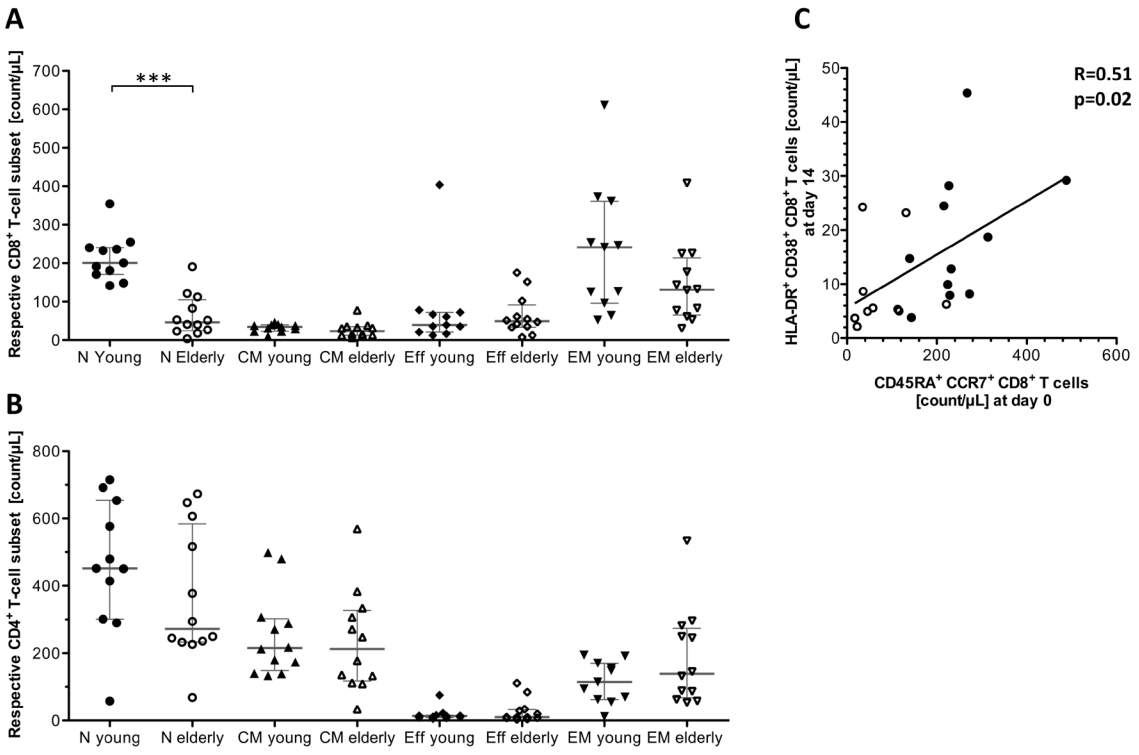
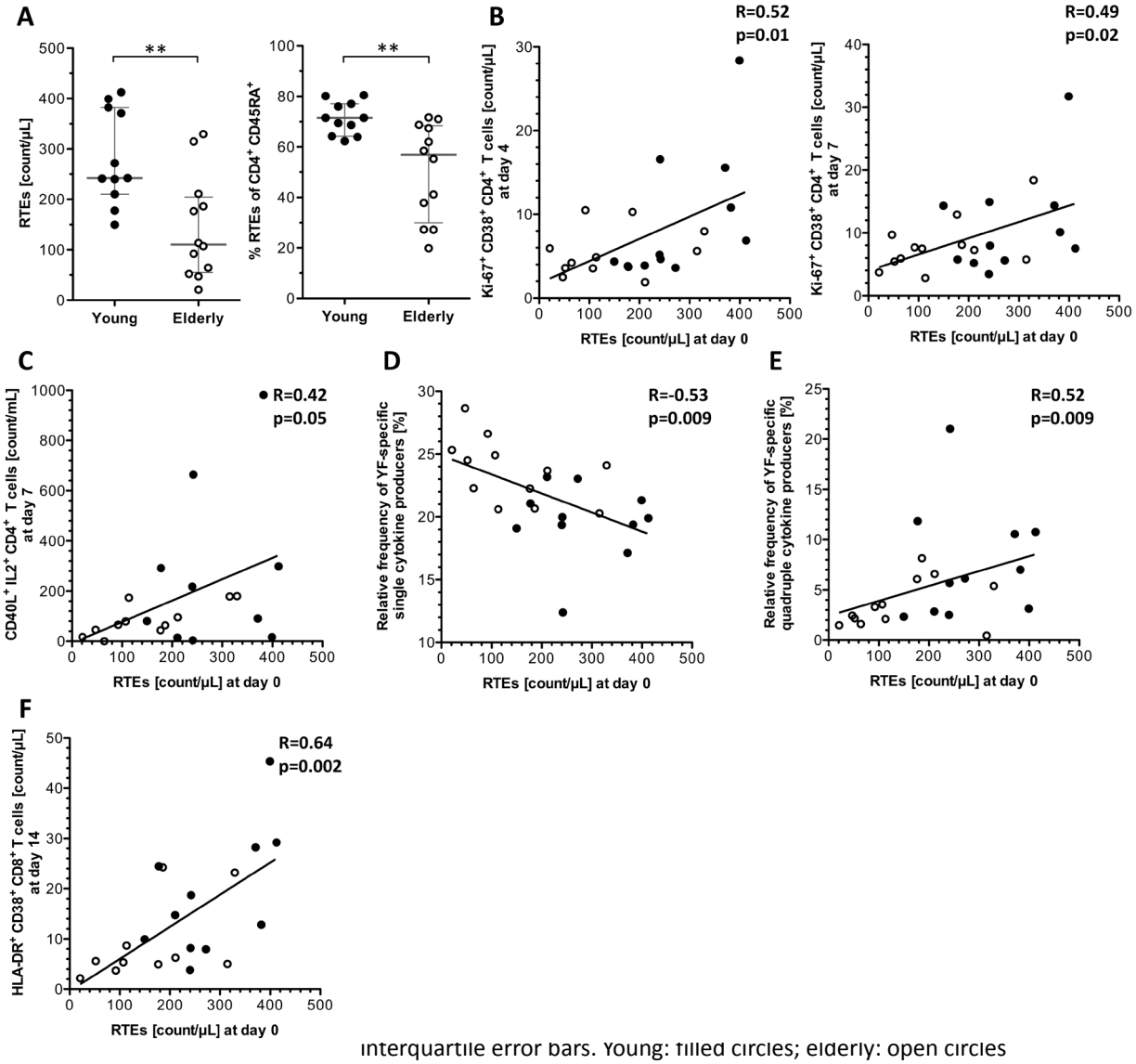


Figure 3.10: Phenotypic composition of the CD8$^+$ and CD4$^+$ T-cell compartment prior vaccination
(**A, B**) Absolute numbers of naive (N), central-memory (CM), effector-memory (EM) and effector (Eff) CD8$^+$ (A) or CD4$^+$ (B) T cells at day 0. Naive CD8$^+$ T cells: p=0.0002 (**C**) Positive correlation between numbers of naive CD8$^+$ T cells prior vaccination and activated HLA-DR$^+$/CD38$^+$ CD8$^+$ T cells at day 14 after vaccination. Lines indicate the median with interquartile error bars. Young: filled symbols; elderly: open symbols

Next, we performed a deep analysis of the naive CD4$^+$ T-cell compartment prior vaccination. It has been previously shown that naive CD4$^+$ T cells can be subdivided by the expression of CD31 into cells having recently emigrated from the thymus (RTE) and cells that have been homeostatically derived (Kimmig *et al.*, 2002). Using the marker CD31 (Chap. 2.2.9.3), we

could demonstrate that elderly had highly significantly lower numbers and frequencies of RTE than young vaccinees before vaccination (Fig. 3.11A). Analyzing possible consequences on subsequent immune mechanisms we found significant positive correlations between numbers of $CD4^+$ RTE at day 0 and the early YF-specific $Ki\text{-}67^+/CD38^+$ $CD4^+$ T-cell response at day 4 and day 7 (Fig. 3.11B), indicating that those individuals with lower RTE counts prior to vaccination have weaker early $CD4^+$ T-cell responses. Similarly, we also found correlations to the YF-specific $CD4^+$ cytokine response in terms of quantity (Fig. 3.11C) and quality (Fig. 3.11, D and E), suggesting that availability of RTE critically governs quantity and quality of YF-specific $CD4^+$ T-cell immunity. Finally, we identified a very strong positive correlation between initial RTE numbers and the YF-specific $CD8^+$ T-cell response at day 14 (Fig. 3.11F), indicating that high RTE numbers prior to vaccination are also important for a good YF-specific $CD8^+$ T-cell response. Collectively, our data demonstrated that high numbers of RTEs, indicative of a broad naive TCR repertoire, were pivotal for a competent YF-specific adaptive immunity.



Interquartile error bars. Young: filled circles; elderly: open circles

3.7.2 Influence of the innate immune system

We also hypothesized that early response signatures of the innate immune system might affect the YF-specific adaptive immune responses. For this, we developed a FACS panel that allowed a comprehensive analysis of major peripheral innate cell types over the entire study course. Our analysis included: Three different DC subsets, such as plasmacytoid DCs (pDCs), myeloid type 1 DCs ($CD11c^+$ mDCs) and myeloid type 2 DCs ($CD141^+$ mDCs), as well as three monocyte subsets: $CD14^{++}/CD16^-$ classical, $CD14^{++}/CD16^+$ intermediate and $CD14^+/CD16^+$ nonclassical monocytes (Chap. 2.2.9.2) and basophils (Fig. 2.2). In addition we assessed neutrophils and eosinophils in 18 of 23 vaccinees by complete blood count (Chap. 2.2.6).

We observed distinct innate response patterns after YF vaccination (Fig. 3.12A-I). Neutrophils, pDCs, $CD11c^+$ mDCs and $CD14^{++}/CD16^-$ classical monocytes were characterized by a significant and transient increase of cell numbers around day 2/4, followed by a drop towards day 7/10 and a secondary increase at the late phase of the response (Day 14 - 21). Other innate cell types, such as eosinophils, basophils and $CD141^+$ mDCs did not show this pronounced primary peak at day 2/4 but a decrease in numbers around day 7/10 and a subsequent re-appearance or even slight peak in the late phase of the response. In contrast to the aforementioned cell types, $CD14^{++}/CD16^+$ intermediate and $CD14^+/CD16^+$ nonclassical monocytes peaked at day 7 after vaccination and subsequently converged to baseline levels. We furthermore found remarkable age-differences in some innate cell subsets: Elderly had highly significantly fewer pDCs and $CD11c^+$ mDCs particularly at the primary peak at day 4. Conversely, they possessed significantly more $CD14^{++}/CD16^+$ intermediate monocytes at day 10 and also higher numbers of $CD14^+/CD16^+$ nonclassical monocytes from day 10 onwards, indicating deferred kinetics of both monocyte subsets in elderly individuals. Interestingly, calculating ratios of classical to nonclassical monocytes revealed very significant differences at all study days between the age groups (Day 4, Fig. 3.12K). Thus, the relative proportion of $CD14^+/CD16^+$ nonclassical monocytes was generally elevated in the monocyte compartment of the elderly.

We applied multivariate analysis to our multiplex innate immune data of day 4 and generated complex, systemic views of our vaccinees (Chap. 2.2.10). The automated cluster analysis (ACA) revealed a relatively continuous distribution of innate immune states among our subjects that could only approximately segregate between different clusters (Fig. 3.13A). However we

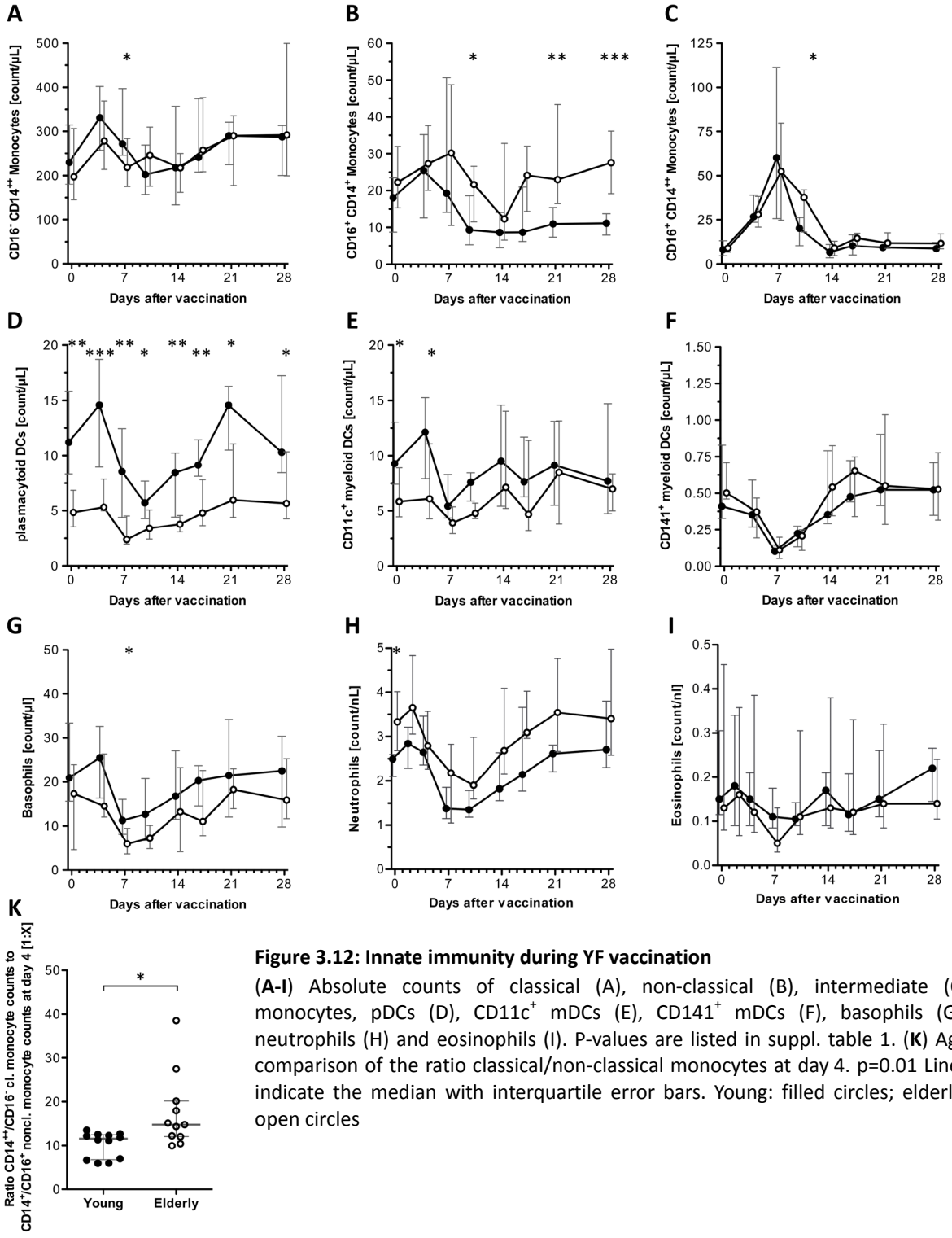


Figure 3.12: Innate immunity during YF vaccination

(**A-I**) Absolute counts of classical (A), non-classical (B), intermediate (C) monocytes, pDCs (D), $CD11c^+$ mDCs (E), $CD141^+$ mDCs (F), basophils (G), neutrophils (H) and eosinophils (I). P-values are listed in suppl. table 1. (**K**) Age comparison of the ratio classical/non-classical monocytes at day 4. p=0.01 Lines indicate the median with interquartile error bars. Young: filled circles; elderly: open circles

observed an accumulation of young vaccinees in clusters 3 and 4 characterized by mostly higher numbers of $CD11c^+$ mDCs, pDCs, basophils and partly lower numbers of $CD16^+/CD14^+$ nonclassical monocytes (cluster 3). In contrast, clusters 1 and 2 were dominated by older vaccinees with intermediate to high levels of $CD16^+/CD14^+$ nonclassical monocytes and lower numbers of $CD11c^+$ mDCs, pDCs and basophils. Exceptional and separated from all other

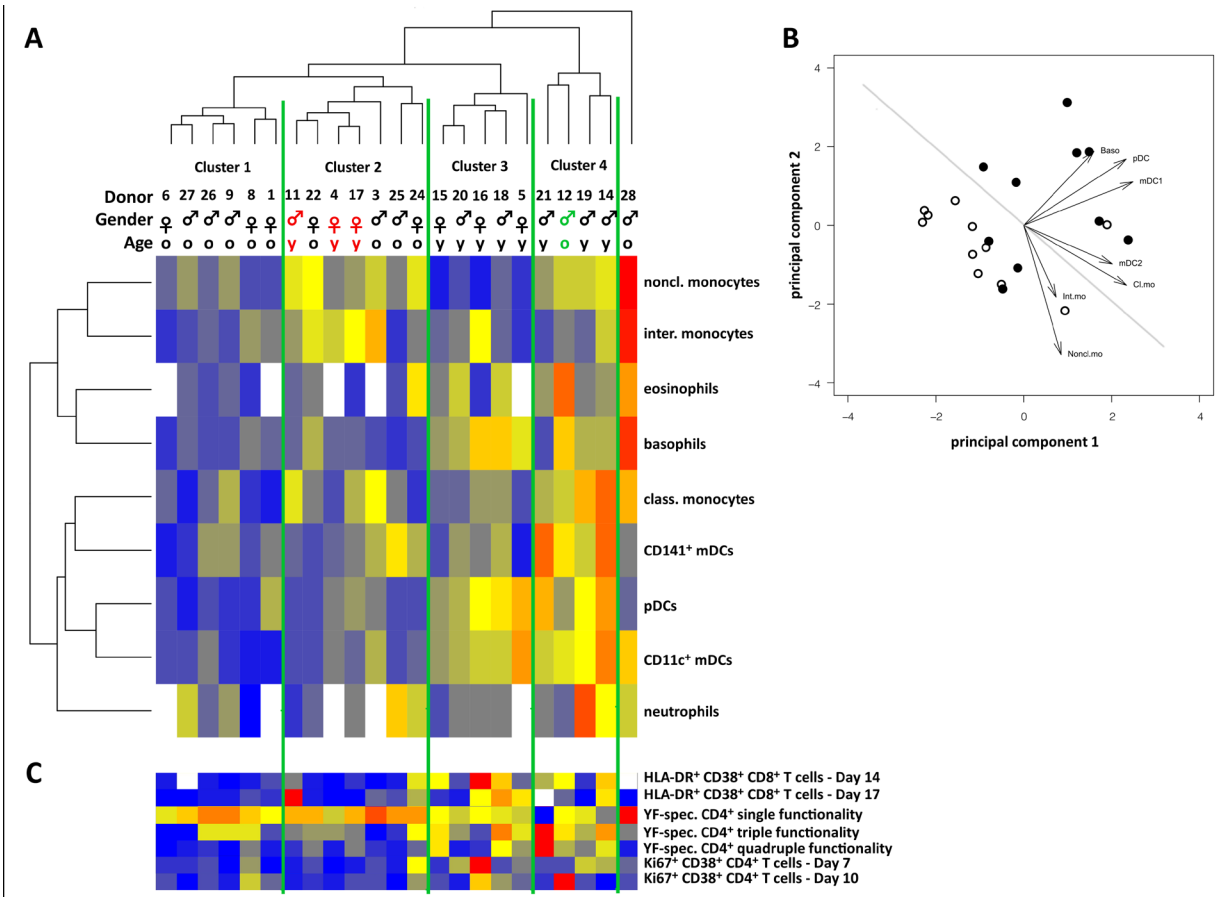


Figure 3.13: Multivariate analysis of innate immunity

(**A**) Automated cluster analysis of innate immunity at day 4 based on whole blood counts of nine major innate cell subsets. Values are scaled and normalized. (**C**) Below, normalized YF response parameters are assigned to the cluster analysis. Dark blue indicates low, red high values, empty fields data not available (**B**) PCA of innate immunity at day 4 based on whole blood counts of seven innate cell subsets. Vector arrows indicate contribution of innate components on PCA (2fold magnified). Young: filled circles; elderly: open circles

vaccinees was donor 28, who had very high amounts of nonclassical and transitional monocytes, basophils and eosinophils. Additionally, we assessed our innate dataset by PCA. Here, we excluded donor 28 from the analysis as he was disproportionally influencing the scaling of the data. As illustrated in fig. 3.13B, YF vaccinees could be segregated into two large clusters following largely the age criterion with only one old and three young misclassified individuals (two-tailed Fisher's test: p=0.007). Thereby, PCA produced a similar classification as the automated cluster analysis, i.e. PCA cluster 1 (mostly elderly) corresponded to ACA cluster 1 and 2, whereas PCA cluster 2 (mostly young vaccinees) was congruent with ACA cluster 3 and 4. Altogether, by multivariate analysis we could comprehensively and unbiasedly compare the young and senescent innate immune system. Similar to the univariate analysis, age was identified as the most determining factor manifested e.g. by decreased DC and increased nonclassical monocyte numbers.

We noticed that the innate immune peak at day 4/7 coincided with the occurrence of mild adverse reactions such as fever, myalgia and erythema. Remarkably, young vaccinees were reporting more frequently side effects than elderly vaccinees (young: 64 %, elderly: 33 %). Grouping vaccinees according to mild adverse symptoms, revealed a clear difference in pDC numbers at day 4, i.e. high numbers of pDCs were characteristic for vaccinees reporting any symptoms (Fig. 3.14A).

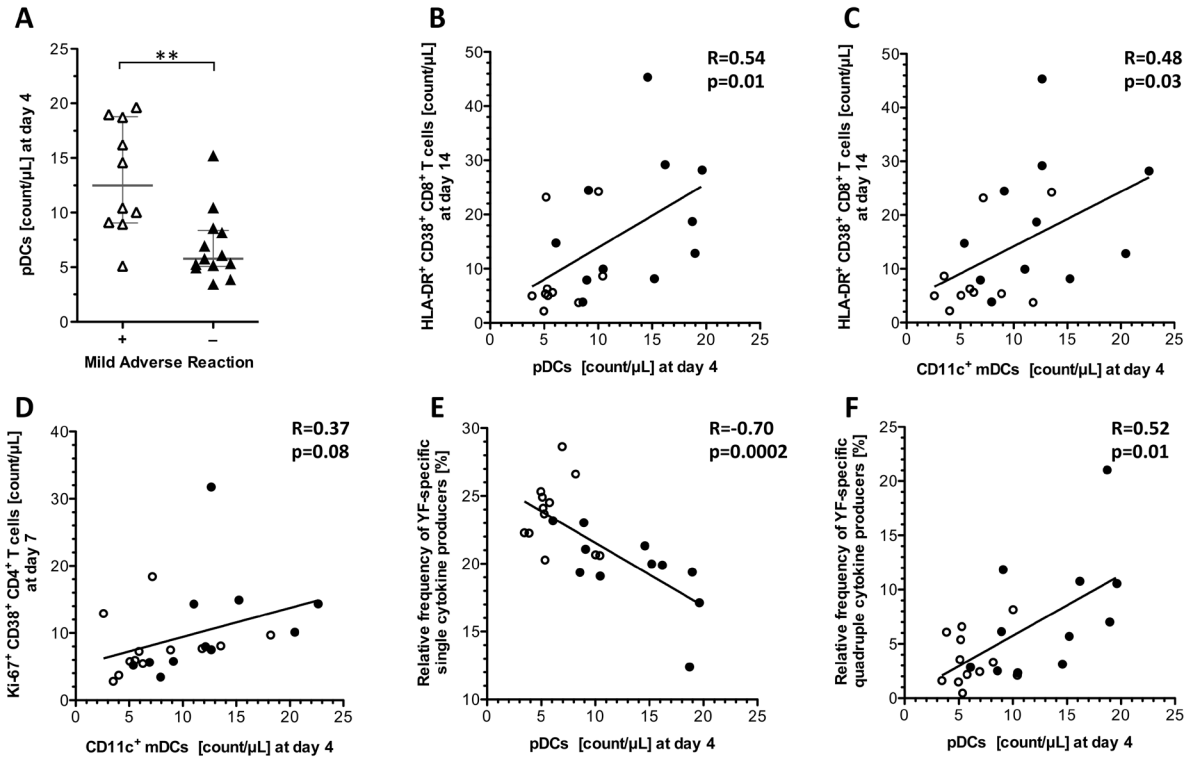


Figure 3.14: Influence of DCs on the YF-specific T-cell response

(**A**) Absolute numbers of pDCs at day 4 in groups with or without occurrence of mild adverse events after vaccination. p=0.005 Without symptoms: filled triangles; With symptoms: open triangles (**B-C**) Positive correlations of absolute numbers of pDCs (B) or CD11c$^+$ mDCs (C) at day 4 with numbers of activated HLA-DR$^+$/CD38$^+$ CD8$^+$ T cells at day 14 after vaccination. For pDCs positive correlations exists also for the CD8$^+$ T-cell response at day 17 and 21. (**D**) Numbers of CD11c$^+$ mDCs at day 4 correlate with numbers of Ki-67$^+$/CD38$^+$ CD4$^+$ T cells at day 7. (**E-F**) Numbers of pDCs at day 4 correlate with qualitative functionality of the YF-specific CD4$^+$ T-cell response. Lines indicate the median with interquartile error bars. Young: filled symbols; elderly: open symbols

We next analyzed whether early innate signatures translated into YF-specific adaptive immune responses. We discovered that numbers of pDCs and CD11c$^+$ mDCs at the peak of innate immunity (day 4) were highly correlative with the specific T-cell response, e.g. both DC subsets positively correlated with numbers of YF-specific HLA-DR$^+$/CD38$^+$ CD8$^+$ T cells at days 14, 17 and 21 (Day 14, Fig. 3.14, B and C). Moreover CD11c$^+$ mDCs also correlated with YF-specific Ki-67$^+$/CD38$^+$ CD4$^+$ T cells in the expansion phase at day 4 and 7 (Day 7, Fig. 3.14D). In line with this, early DC responses also translated positively into YF-specific CD4$^+$ cytokine responses at day 7 and 10 (not shown) and were significantly related to polyfunctionality of

YF-specific $CD4^+$ T cells (Fig. 3.14, E and F). We further connected cluster analysis of innate cell subsets at day 4 with major adaptive response parameters (e.g. acutely activated $CD4^+$ and $CD8^+$ T cells) that were scaled and color-coded for better visualization (Fig. 3.13C). The distribution of some of these parameters amongst the clustered vaccinees followed very interesting patterns: For example YF-specific HLA-DR^+/$CD38^+$ $CD8^+$ T cells at day 14 and 17 were clearly higher in clusters 3 and 4 than in cluster 1 and 2. The same could be observed for $CD4^+$ cytokine polyfunctionality (3 and 4 functions), whereas the opposite was true for $CD4^+$ single functionality. It was of note that these patterns did not solely follow the age criterion, but the true individual innate immune state. For example donor 12 was grouped despite its age to cluster 4 and demonstrated equally high levels of e.g. YF-specific HLA-DR^+/$CD38^+$ $CD8^+$ T cells as young vaccinees of cluster 3 and 4. Conversely, two of three young vaccinees (4 and 17), who belonged to cluster 2, showed similar low levels of YF-specific HLA-DR^+/$CD38^+$ $CD8^+$ T cells as elderly subjects of cluster 1 and 2. Consistently, we could visualize these associations also in our PCA when we overlaid median-segregated YF-specific response signatures (Suppl. fig. 2A-D). Thus multivariate analysis strongly supported the correlations observed by univariate analysis demonstrating a close connection of innate signatures particularly of pDCs and $CD11c^+$ mDCs with quantity and quality of the YF-specific adaptive T-cell response.

3.7.3 Influence of early immune cell migration

Our multiplex quantitative immune assessment enabled us to investigate the temporospatial distribution and composition of immune cells and their subsets in peripheral blood. Plotting absolute cell numbers against time revealed distinct kinetic patterns of each immune subset. As shown in fig. 3.15A-C peripheral numbers of B and T cells highly significantly dropped from day 0 to day 7. This drop was transient, as numbers quickly reached or partially even exceeded pre-vaccination cell counts. Furthermore, we could observe the same characteristic drop in some, but not all subpopulations of B, T and NK cells. For example, peripheral numbers of naive and CM T cells pronouncedly dropped from day 0 to day 7, whereas EM and Eff $CD4^+$ and $CD8^+$ T-cell numbers did not significantly change (Fig. 3.15D-G; Suppl. fig. 3A-C). In the B-cell compartment, numbers of naive and memory cells transiently dropped as well (Suppl. fig. 3, F and G), whereas in the NK-cell compartment (Chap. 2.2.9.5) only $CD56^{bright}$ cells showed this behavior (Suppl. fig. 3, D and E). Interestingly, these subset-specific cell migration

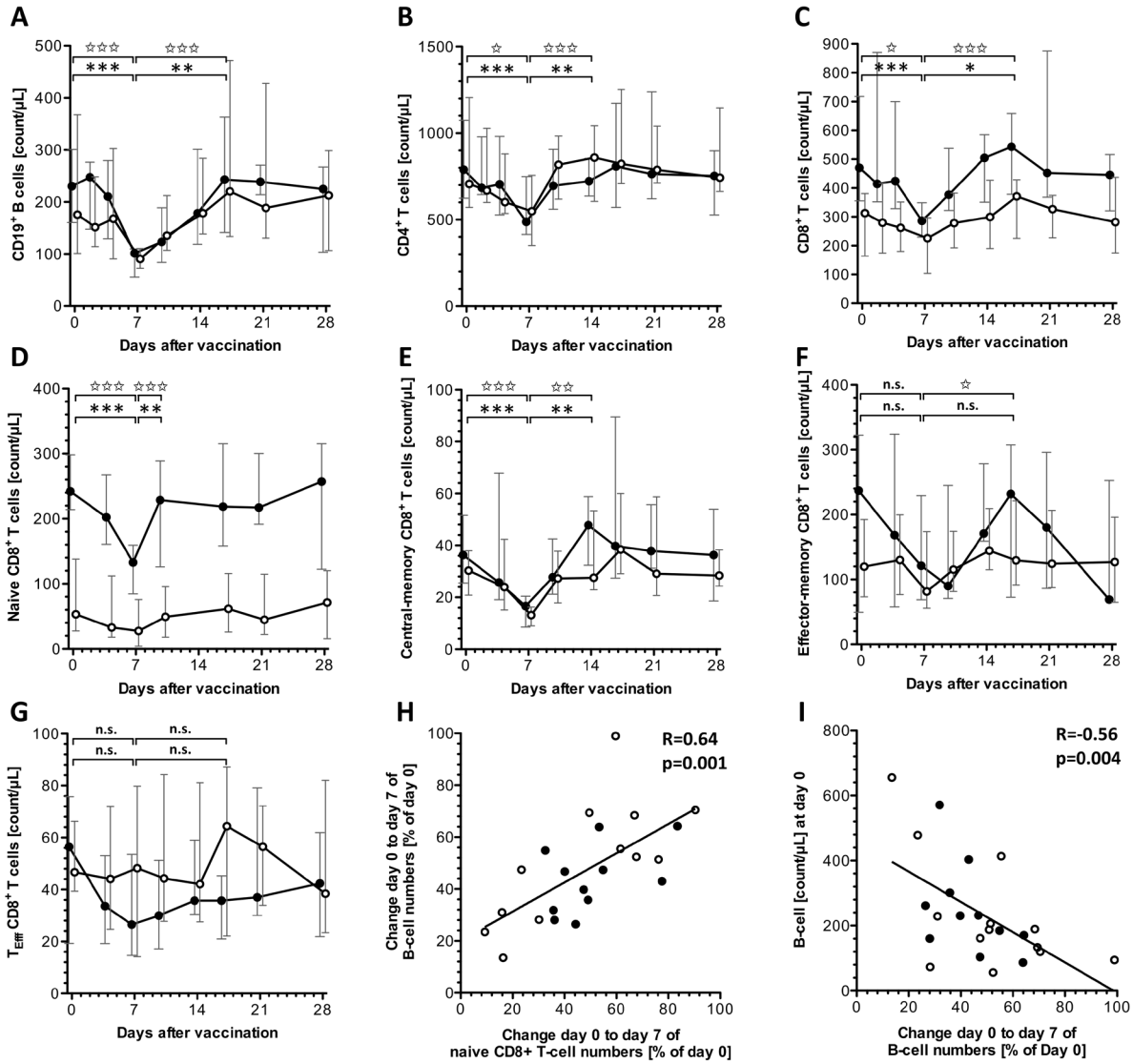


Figure 3.15: Temporospatial distribution and composition of immune cells in peripheral blood
(**A-G**) Absolute cell counts of (A) B cells, (B) $CD4^+$, (C) $CD8^+$, (D) naive $CD8^+$, (E) CM $CD8^+$, (F) EM $CD8^+$ and (G) effector $CD8^+$ T cells in peripheral blood in the course of the vaccine-induced immune response. Lines indicate the median with interquartile error bars. Wilcoxon paired signed rank tests were performed for each age group. Asterisks indicate significances in young and stars in elderly vaccinees. P-values are listed in suppl. table 2. (**H**) "Day 0/day 7 drops" correlate with each other, exemplified by the change of $CD8^+$ T-cell numbers versus B-cell numbers from day 0 to day 7. (**I**) "Day 0/day 7 drops" correlate with initial numbers of the respective immune cell subset. As an example numbers and changes of B cells are shown. Young: filled circles; elderly: open circles

patterns extensively correlated with each other, e.g. a strong reduction of naive $CD8^+$ T-cell numbers was associated with a steep B-cell drop (Fig. 3.15H). In contrast, we could not find any influence of age (not shown). Hypothesizing that innate immune signatures might provoke more pronounced migration patterns, we correlated innate cell numbers and early innate cell increases with the level of decrease of T, B and NK-cell subset numbers from day 0 to day 7. However we could not detect any significant association (not shown). In contrast, we found highly significant correlations between pre-vaccination cell numbers and the respective cell number drop of each subset, i.e. individuals with higher numbers of e.g. peripheral B cells

prior vaccination experienced a more pronounced B-cell drop than vaccinees with lower initial B-cell counts (Fig. 3.15I). We further hypothesized that the early cell number drop may influence subsequent adaptive immune responses. However, the correlation analysis revealed no significant associations with YF-neutralizing antibody titers, plasmablast responses or YF-specific T-cell responses (not shown). Collectively, we identified a remarkable YF-induced early cell migration pattern characteristic for certain B, T, and NK-cell populations that was strongly influenced by respective initial cell counts but not by age.

3.7.4 Influence of chronic infections

Chronic viral infections such as CMV and EBV infections are thought to constantly challenge and shape the immune system of their host. Hence, we obtained the CMV and EBV status of the vaccinees and investigated the influence on the YF-specific response (Chap. 2.2.7). As indicated in fig. 3.16A, we detected in all vaccinees EBV-specific IgG antibody titers that did not differ between both age groups. On the contrary, CMV-specific IgG antibodies were found in 58 % elderly and 45 % young vaccinees. CMV IgG titers were equal in both CMV^+ age groups (Fig. 3.16B). We further assessed CMV and EBV-specific $CD4^+$ and $CD8^+$ T-cell responses before vaccination (Chap 2.2.9.8). All vaccinees showed an EBV-specific $CD8^+$ T-cell response (Fig. 3.16C). With respect to CMV, 58 % old and 45 % young vaccinees had detectable specific $CD8^+$, and 67 % old and 45 % young detectable specific $CD4^+$ T cells (Fig. 3.16, D and E). Similar to the EBV/CMV serostatus we did not observe any age difference in the EBV/CMV-specific cellular responses. The overlap between serological and cellular CMV states was nearly perfect as illustrated in fig. 3.16, F and G, cross-validating our assessment of CMV response. Interestingly, EBV and CMV IgG serostatus (CMV^+ individuals only) exhibited a high degree of concordance (Fig. 3.16H), which becomes important in the interpretation of the YF follow up study (Chap. 3.8).

We further investigated whether CMV/EBV status affects the baseline immune constitution of vaccinees. From all investigated innate cell subsets (Chap. 2.2.9.2) only numbers of $CD11c^+$ and $CD141^+$ mDCs were negatively associated with EBV IgG serostatus (Fig. 3.17, A and B). Also, it seemed that the CMV status might influence mDC and monocyte development or distribution as CMV positive vaccinees had a significantly higher ratio of $CD11c^+$ and $CD141^+$ mDC numbers to classical monocytes (Fig. 3.17C and not shown). Regarding phenotypic T-cell composition we identified an association of EBV/CMV status with Eff, EM

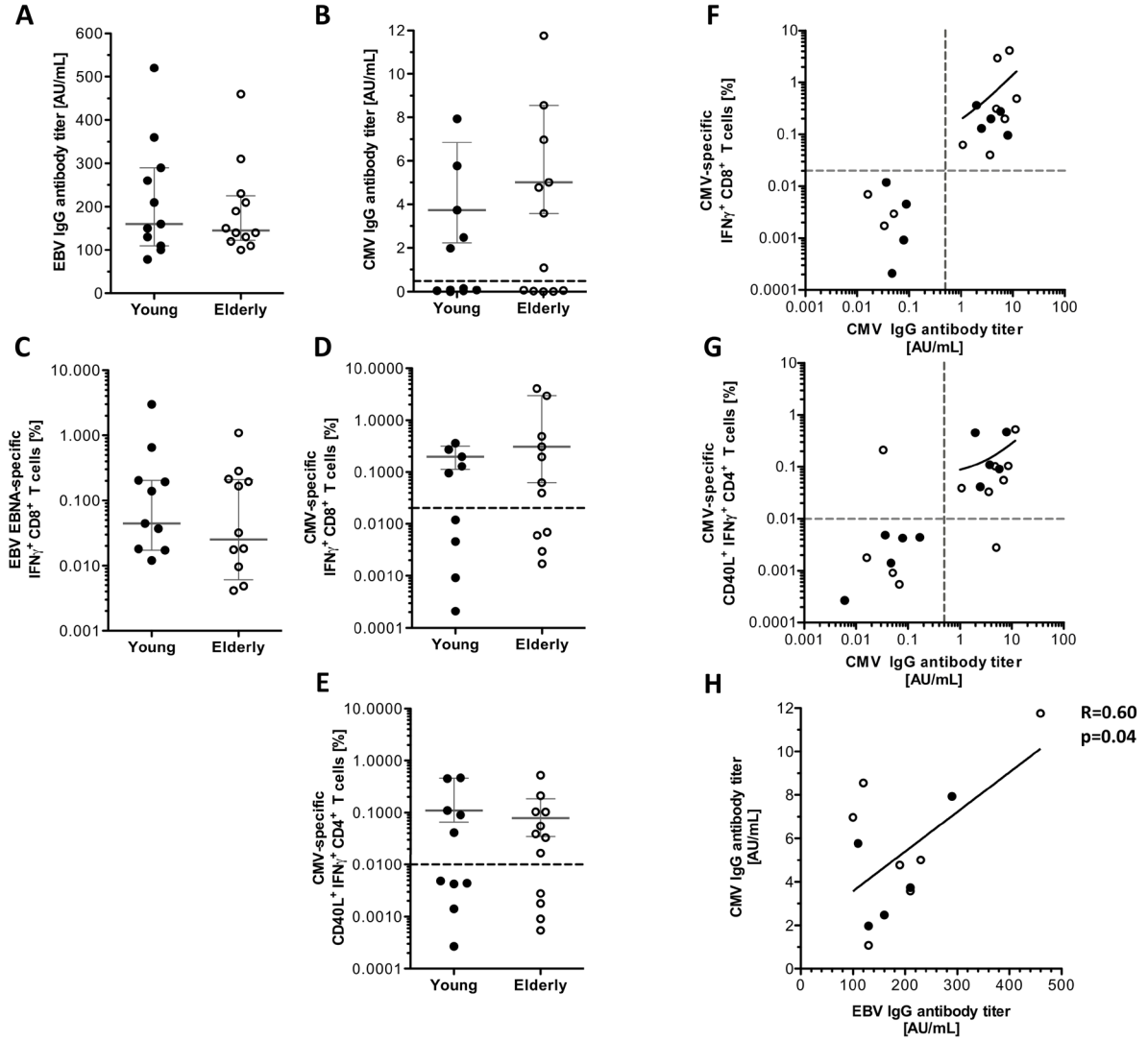


Figure 3.16: EBV and CMV status of YF vaccinees prior vaccination

(**A**) EBV and (**B**) CMV IgG sero status of young and elderly vaccinees. Frequencies of (**C**) EBV-specific and (**D**) CMV-specific $CD8^+$ T cells after antigen-specific re-stimulation. (**E**) Frequencies of CMV-specific $CD4^+$ T cells after CMV-specific re-stimulation. No significant age-specific differences were observed. (**F, G**) High congruence between CMV sero-status and measurements of CMV-specific (F) $CD8^+$ and (G) $CD4^+$ T cells. (**H**) CMV and EBV IgG sero titers correlate with each other (only CMV^+ donors). Dashed lines indicate assay-specific cut-off values for CMV positivity. Note that due to the logarithmic scale zero values cannot be displayed. Lines indicate the median with interquartile error bars. Young: filled circles; elderly: open circles

and CM frequencies. For example, individuals with high EBV IgG titers exhibited $CD8^+$ and $CD4^+$ T-cell compositions skewed towards higher Eff and lower CM T-cell frequencies (Fig. 3.17, D and E). Some individuals having been CMV positive showed increased frequencies of Eff $CD8^+$ T cells and EM $CD4^+$ T cells (Fig. 3.17F and not shown). Generally, compositional changes observed as percentage could be found also in absolute cell numbers of T-cell subsets. Importantly, CMV/EBV status affected neither frequencies nor numbers of naive $CD4^+$ and $CD8^+$ T cells or $CD4^+$ RTEs in our study.

We also explored the YF-specific adaptive immune response for any correlations with

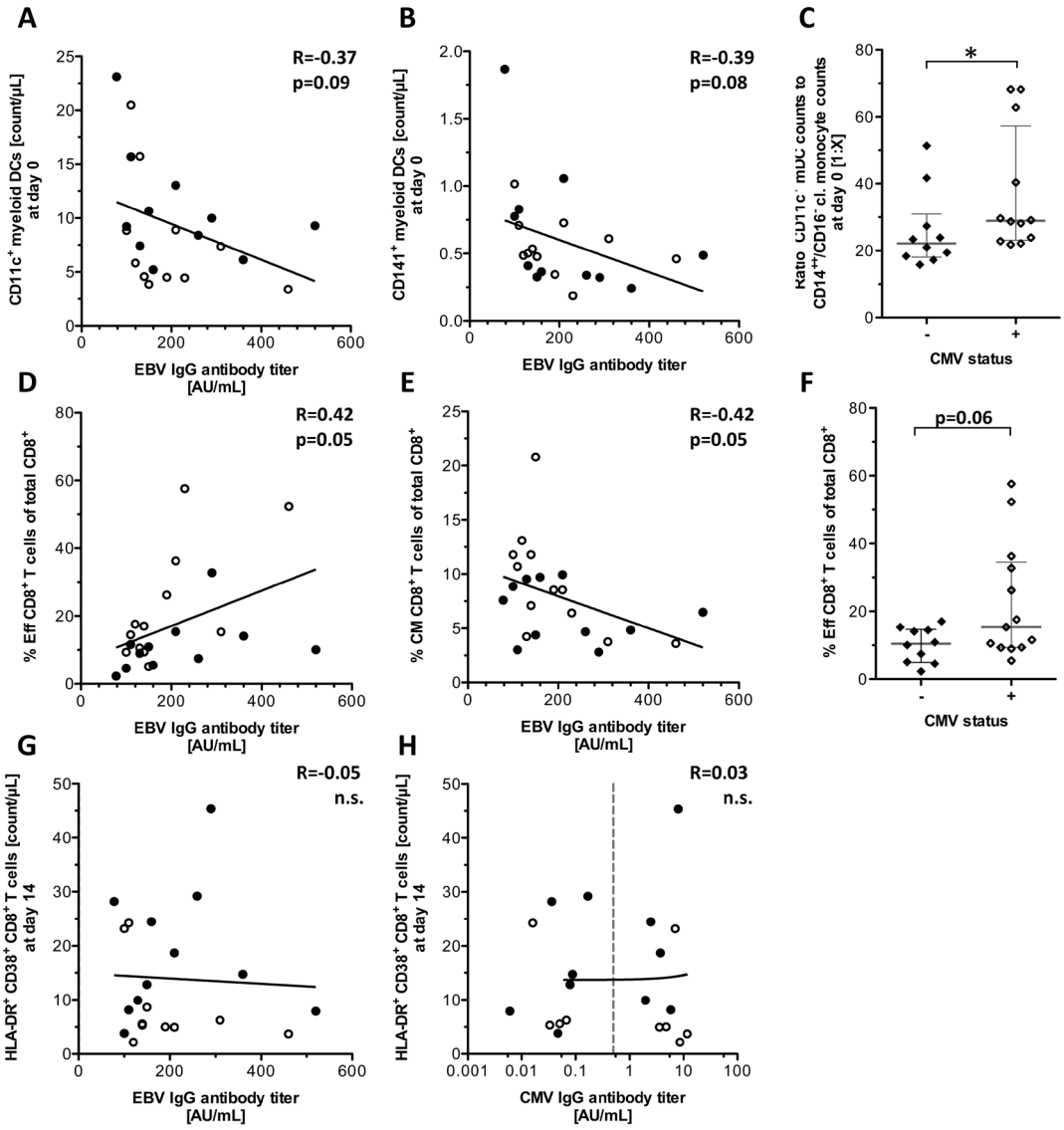


Figure 3.17: Influence of EBV and CMV status on baseline immune properties and acute YF-specific adaptive immunity

Negative correlation of EBV IgG sero titers with absolute numbers of (**A**) $CD11c^+$ mDCs and (**B**) $CD141^+$ mDCs at day 0. (**C**) Significantly increased ratio of $CD11c^+$ mDCs to classical monocytes at day 0 in CMV positive vaccinees. p=0.04 (**D, E**) EBV IgG titers are associated with (D) increased frequencies of effector and (E) decreased frequencies of CM $CD8^+$ T cells at day 0. (**F**) Some CMV positive vaccinees exhibit increased frequencies of effector $CD8^+$ T cells at day 0. (**G, H**) Examples showing that (G) EBV IgG titers and (H) CMV IgG titers do not correlate with activated $HLA\text{-}DR^+/CD38^+$ $CD8^+$ T cells at day 14 after vaccination. Lines indicate the median with interquartile error bars. Dashed line indicates assay-specific cut-off values for CMV positivity. Young: filled circles; elderly: open circles; CMV IgG negative: filled squares, CMV IgG positive: open squares

CMV/EBV status, however we could neither discover any significant impact on YF-neutralizing antibody titers (not shown) nor on quantity and quality of YF-specific $CD4^+$ or $CD8^+$ T cells (Fig. 3.17, G and H). Altogether, our results indicate that CMV and EBV infection affected steady state innate immunity and T-cell constitution in terms of memory/effector phenotypic composition. However, it was not possible to prove any influence on the naive constitution or the YF-specific adaptive immune response.

3.8 Long-term YF-specific immunity

So far our analysis was focused on the acute phase after YF vaccination. However, establishment of long-lasting immunity conveying protection against dangerous YF wild type infection was another important aspect that we wanted to address in our study. For this, we were able to re-invite 8 of 11 young and of 11 of 12 elderly study participants 20 to 35 months after YF vaccination and assessed their YF-specific humoral and adaptive immunity. As indicated in fig. 3.18A, all vaccinees still demonstrated protective titers of YF-neutralizing antibodies, which did not differ between the age groups. In comparison to day 28, titers decreased in most of the individuals (Fig. 3.18B) regardless of age (Fig. 3.18C). We next searched for factors influencing long-term YF-specific humoral immunity, but identified neither associations with acute YF-specific humoral nor cellular immune responses. However, we found an unexpected association between the decline of YF-neutralizing titers and EBV-specific IgG antibody titers (Fig. 3.18D), i.e. individuals with high EBV IgG titers had an improved stability of long-term humoral protection against YF.

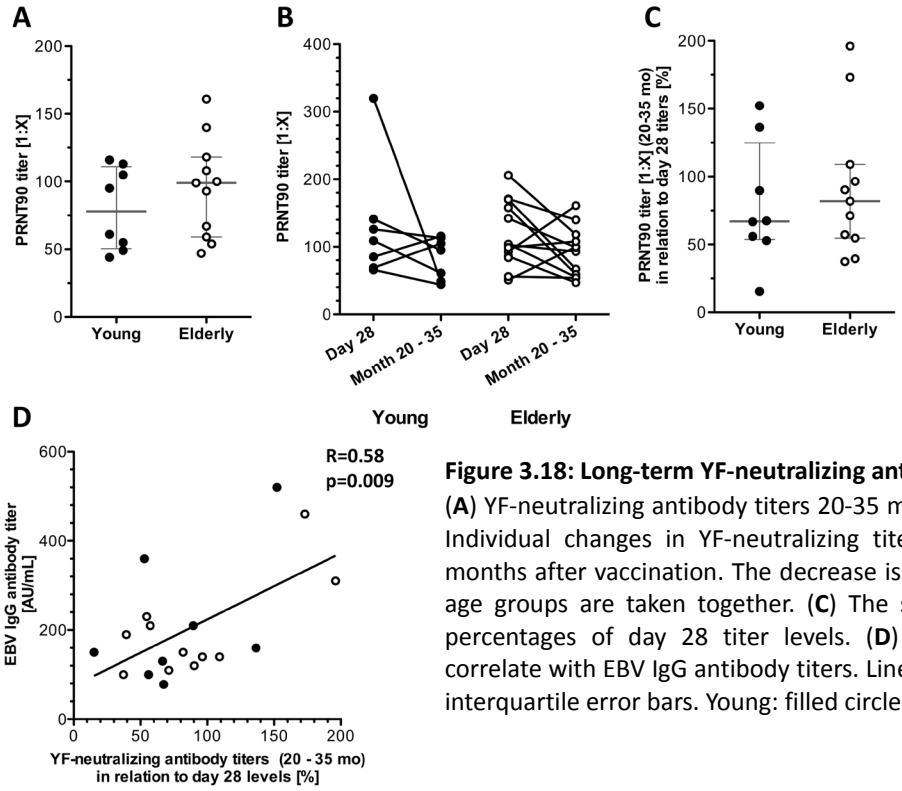


Figure 3.18: Long-term YF-neutralizing antibody response
(**A**) YF-neutralizing antibody titers 20-35 months post vaccination. (**B**) Individual changes in YF-neutralizing titers from day 28 to 20-35 months after vaccination. The decrease is significant (p=0.03) if both age groups are taken together. (**C**) The same changes depicted as percentages of day 28 titer levels. (**D**) These changes positively correlate with EBV IgG antibody titers. Lines indicate the median with interquartile error bars. Young: filled circles; elderly: open circles

Furthermore, we tracked in ten HLA-A0201$^+$ vaccinees IFNγ-producing YF-specific CD8$^+$ T cells after peptide stimulation. Numbers of these cells decreased about 7 fold in comparison to day 28, but still were clearly detectable (Fig. 3.19, A and B). We could not reveal any significant age difference in terms of long-term cell numbers and contraction (Fig. 3.19C).

However, comparing the acute YF-specific IFNγ$^+$ CD8$^+$ T-cell response with long-term immunity we could identify a strong, positive relationship between acute and long-term YF-specific CD8$^+$ T-cell levels (Fig. 3.19D). We further noticed that strong cellular CMV-specific T-cell responses tended to be associated with a deficient long-term stability of YF-specific CD8$^+$ T cells (Fig. 3.19E). Other parameters of the acute phase, such as cell numbers of various innate subsets or phenotypic composition of the T-cell compartment, did not seem to correlate with the long-term persistence of YF-specific CD8$^+$ T cells.

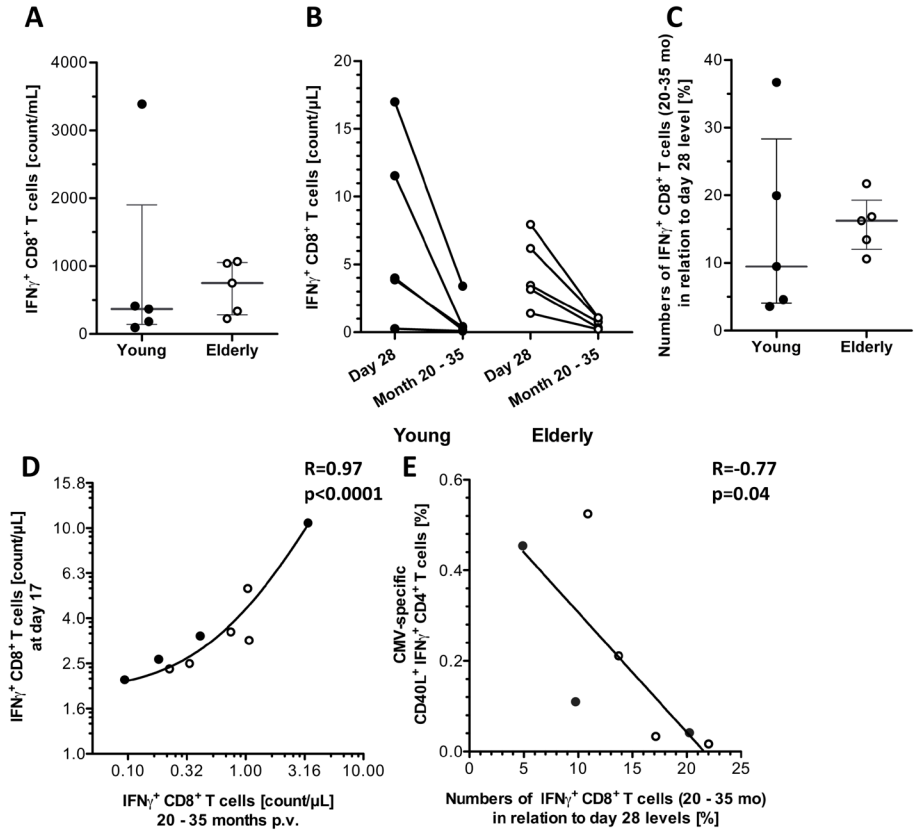


Figure 3.19: Long-term YF-specific CD8$^+$ T-cell response

(**A**) Absolute numbers of YF-specific CD8$^+$ T cells 20-35 months post vaccination. (**B**) Individual decreases of YF-specific CD8$^+$ T-cell numbers from day 28 to 20-35 months after vaccination. (**C**) The same decreases depicted as percentages of day 28 levels. (**D**) Magnitudes of YF-specific CD8$^+$ T-cell responses at day 17 and 20-35 months post vaccination correlate with each other. Similar correlations were found with day 21 and 28 and with percentages of the respective days. (E) Long-term stability of YF-specific CD8$^+$ T-cell response correlates with percentage of CMV-specific (**E**) CD4$^+$ and CD8$^+$ (not shown) T cells. Lines indicate the median with interquartile error bars. Young: filled circles; elderly: open circles

We additionally analyzed the long-term YF-specific CD4$^+$ T-cell response by vaccine stimulation and ICS. All analyzed subjects demonstrated detectable numbers of YF-specific CD4$^+$ T cells, expressing at least one cytokine (Fig. 3.20A-D). Remarkably, numbers of IFNγ-producing YF-specific CD4$^+$ T cells were significantly lower in the elderly cohort, whereas numbers of other cytokine producers did not differ. Three elderly individuals even had no detectable IFNγ$^+$ CD4$^+$ T cells upon vaccine re-stimulation. In comparison to day 28, most of the vaccinees

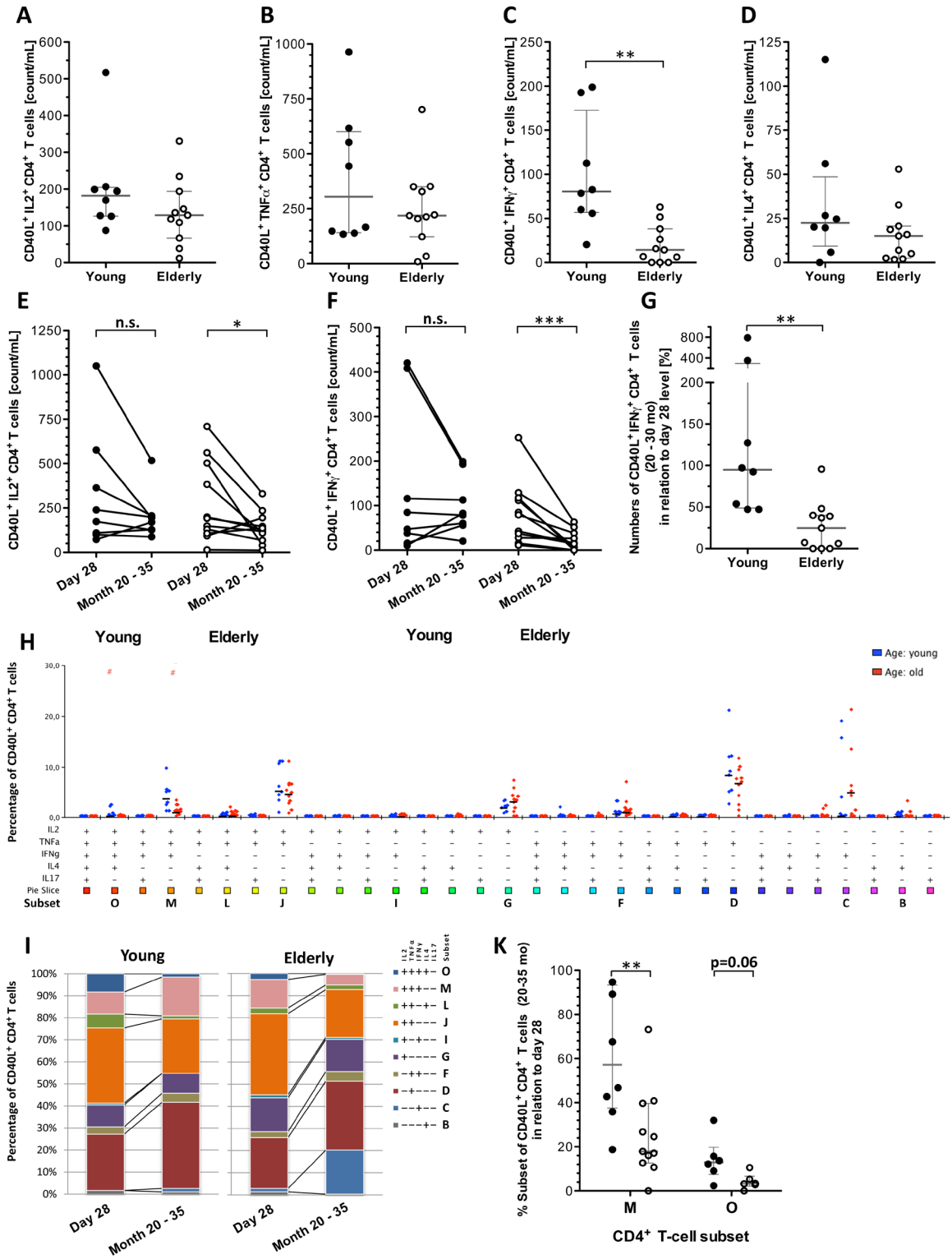


Figure 3.20: Long-term YF-specific CD4$^+$ T-cell response

(**A-D**) Absolute numbers of YF-specific CD4$^+$ cytokine producing cells 20-35 months post vaccination. CD40L$^+$ IFNγ$^+$ CD4$^+$ T cells: p=0.002 (**E-F**) Individual changes of YF-specific (E) CD40L$^+$/IL2$^+$ and (F) CD40L$^+$/IFNγ$^+$ CD4$^+$ T-cell numbers from day 28 to 20-35 months after vaccination. (Wilcoxon matched pairs test: Elderly CD40L$^+$/IL2$^+$ p=0.03; Elderly CD40L$^+$/IFNγ$^+$ p=0.001) (**G**) Changes in CD40L$^+$/IFNγ$^+$ CD4$^+$ T-cell numbers depicted as percentage of day 28 levels. p=0.002 (**H**) Age comparison of the qualitative YF-specific CD4$^+$ T-cell response 20-35 months after vaccination. Young: blue dots; Old: red dots. Black bars depict medians in each subset. Significant differences are indicated by hashes. (**I**) Changes in the YF-specific qualitative CD4$^+$ composition (major subsets) from day 28 to 20-35 months after vaccination in both age groups. (**K**) Disproportional decrease of subsets M and O in elderly 20-35 months after vaccination when compared to day 28 levels. Subset M: p=0.007 Lines indicate the median with interquartile error bars. Young: filled circles; elderly: open circles

showed decreasing YF-specific $CD4^+$ T-cell levels (about 1.2 fold for $CD40L^+$ $IL2^+$ $CD4^+$ T cells) (Fig. 3.20, E and F). Notably, IFNγ-producing YF-specific $CD4^+$ T cells disproportionally declined in the elderly cohort (Fig. 3.20G). Multiparametric analysis by SPICE revealed the long-term qualitative composition of YF-specific $CD40L^+$ $CD4^+$ T cells (Fig. 3.20H). 20 to 35 months after vaccination the immune response was still dominated by certain subpopulations, such as TNFα single producers (subpop. D) or TNFα/IL2 double producers (subpop. J). At this very late time point significant age differences were only observed in subpop. M and O, i.e. elderly possessed lower relative frequencies of TNFα/IL2/IFNγ triple and TNFα/IL2/IFNγ/IL4 quadruple producers than young vaccinees. In comparison to the qualitative composition at day 28 relative proportions of some subpopulations (e.g. C and D) increased and other subpopulations (e.g. J and O) decreased (Fig. 3.20I). Whereas most of these changes were insignificant between both age groups, relative frequencies of polyfunctional subpop. M and O disproportionally declined in the elderly (Fig. 3.20K). As both subsets were also expressing IFNγ, this qualitative reduction of $CD4^+$ polyfunctionality in long-term immunity substantially affected the observed decreased $CD4^+$ IFNγ production in the elderly.

Searching for immune signatures of the acute phase influencing long-lasting YF- specific $CD4^+$ T-cell immunity, we discovered very significant correlations between acute and long-term $CD4^+$ T-cell responses (Fig. 3.21A). Thus and in analogy to long-term YF-specific $CD8^+$ immunity, individuals with strong acute YF-specific $CD4^+$ responses demonstrated 20 - 30 months later increased YF-specific $CD4^+$ memory responses, which was true for all measured cytokines. Since robust acute YF-specific $CD4^+$ T-cell responses were strongly related to high numbers of pDCs, $CD11c^+$ mDCs and RTEs, we could as well reveal these underlying associations in long-term YF- specific $CD4^+$ T-cell immunity (Fig. 3.21, B and C). Regarding CMV/EBV status of the vaccinees we could not identify any effect on long-term YF-specific $CD4^+$ T-cell immunity, contrasting the results found in $CD8^+$ T cells.

Collectively, our analysis demonstrated presence of protective long-lasting YF-specific humoral and cellular immunity in all vaccinees regardless of age. We could show that up to 3 years post vaccination the magnitude of remnant cellular immunity was largely determined by strength of the previous acute cellular response. Moreover chronic infection with CMV and EBV seemed to differentially influence stability of YF-specific humoral and $CD8^+$ T cell responses.

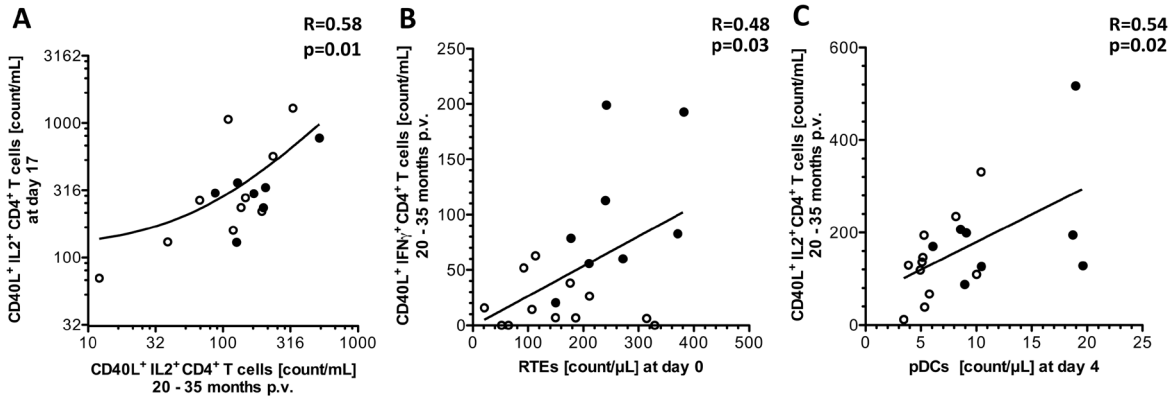


Figure 3.21: Correlation of long-term YF-specific CD4$^+$ T-cell immunity with the acute YF response

(**A**) YF-specific long-term CD4$^+$ T-cell responses significantly correlate with YF-specific acute CD4$^+$ T cell responses from day 7 to 28. Shown is the correlation with values from day 17. (**B**) Correlation between initial numbers of CD4$^+$ RTE and long-term numbers of CD40L$^+$ IFNγ$^+$ CD4$^+$ T cells. (**C**) Correlation between numbers of pDCs at day 4 and long-term numbers of CD40L$^+$ IL2$^+$ CD4$^+$ T cells. Young: filled circles; elderly: open circles

4. Discussion

4.1 Challenges of investigating primary immune responses in humans

As entire primary acute immune responses have never been investigated in such detail before in human elderly, we conceptualized a study with an exploratory, discovery-driven character and with very general initial assumptions. Given also the ethical restrictions for live vaccinations in human elderly, our approach was determined by some limitations, that one has to become aware of, before discussing the results.

First, our study was restricted in the upper age of the elderly cohort, due to ethical concerns. With a reporting rate of 1-2.3 per 100,000 vaccinations, persons aged more than 60 years are at 4-13 fold higher risk than young adults to develop serious YEL-AVD (Monath, 2012) and therefore require careful risk-benefit analysis when YF-vaccinated in Germany. In other countries YF vaccination of this age group is even contraindicated. For this reason, we recruited most of the elderly volunteers in the range of 55-65. Only 3 participants were in the range of 65-70. Although our aged cohort was hence at the ethically permitted most upper age border for YF vaccination, it was relatively "young" in comparison to other studies in the field of immunological aging, usually focusing on persons being significantly older than 70 (Pawelec, 2012).

Second, and mostly due to our very restrictive recruitment, our study was relatively small with only 26 enrolled participants. Of these, we had to omit two vaccinees due to pre-existing tick-borne encephalitis virus-specific antibodies originating perhaps from natural, occult infection (Gustafson *et al.*, 1993; Kaiser *et al.*, 1997; Hunfeld *et al.*, 1998). As these antibodies cross-react with YFV (Allwinn *et al.*, 2002; Holzmann, 2003; Litzba *et al.*, 2014), they potentially impede the generation of a truly primary immune response. From all published YF vaccination studies, this is the first study that did not solely rely on vaccination records, but in addition applied laboratory testing for pre-existing cross-reactive antibodies. The exclusion of two participants confirmed the necessity of this thorough pre-screening.

Third, as a consequence of the small sample number, statistical analysis was complicated by low statistical power. A further difficulty was the high individual variability, inherent to human studies. To account for this, we applied very robust statistics, i.e. medians, interquartile ranges and two-sided Mann-Whitney-U test were used. Furthermore, we performed the study in three independent rounds and avoided further sub-grouping, which would make proper evaluation impossible. Due to the small sample size we did not adjust the significance level for

multiple testing. Since we applied correlation analyses one should be aware that correlation does not necessarily imply causality, but e.g. could instead involve a third unknown variable. To increase certainty, we considered correlations usually only when they were observed (at least by trend) on consecutive days.

And fourth, the study design was exploratory. For example, the blood drawing scheme was assumed from experience with young YF vaccinees, thought it was unclear whether this would apply also to old YF vaccinees who might have a different time course. As we measured YF responses in the elderly for the first time and for this analyzed leukocyte subsets not even having been assessed in such a context in young vaccinees, we started mostly with very general initial hypotheses, e.g. that elderly YF vaccinees have weaker or slower immune responses in comparison to young adults.

In view of such premises and the fact that we obtained more than 16,000 individual data points, it should be noted that most results require validation from large confirmatory studies. Nevertheless, some results could be already demonstrated in animal studies or fit well to long-suspected assumptions. Thus this study provides ample interesting findings and associations, providing the basis for further experimental human aging studies.

4.2 Late viremia and reduced neutralizing antibody titers in elderly vaccinees

The first key finding of our study was that elderly vaccinees did not only develop 1.2 fold more often a detectable serum viremia but also that the viremia occurred significantly later than in young adults. In particular remarkable were four old vaccinees with peak viremia on day 7 or 10, which is a very unusual feature as viremia in young adults occurs between day 2 to 7 (Reinhardt *et al.*, 1998; Monath *et al.*, 2003; Akondy *et al.*, 2015). Also a recent YF modeling paper reports that peak viral load in the blood in young adults is reached on average on day 5 (Le *et al.*, 2014). Our observations were in strong concordance with data from Roukens *et al*., the only yet published aging study investigating the acute response after YF vaccination (Roukens *et al.*, 2011), who report for their old cohort an increased frequency of serum viremia as well as virus detectable in blood for longer periods and on late time points (day 10). Late occurrence of viremia seems to be a detrimental feature and an indication for ineffective virus control as late viremia has been also reported for a 64-year-old vaccinee, who experienced YF-SAE (Pulendran *et al.*, 2008) and in aged mice models, challenged with sublethal, primary flu or LCMV infections (Murasko and Jiang, 2005). Thus, we propose that

late viremia in the elderly is a consequence of impaired viral clearance, possibly due to diminished anti-viral immune effector mechanisms.

Indeed and in line with Roukens *et al.* (Roukens *et al.*, 2011), we observed reduced YF-neutralizing antibody titers in elderly vaccinees between days 14 to 21. Of note, both age groups reached by day 28 comparable neutralizing titers, which were maintained equally in both groups 2-3 years post vaccination, which is in agreement with results from other large YF vaccination trials (Monath *et al.*, 2005; Vratskikh *et al.*, 2013). Interestingly, in primary TBE vaccination significantly lower neutralizing antibody titers were achieved in aged cohorts (Hainz *et al.*, 2005; Weinberger *et al.*, 2010; Stiasny *et al.*, 2012), suggesting that underlying mechanisms might differ between live-virus vaccinations, such as against YFV, imperatively requiring complete virus elimination, and inactivated whole-virus vaccinations, such as against TBE virus.

In contrast to other YF studies, we additionally analyzed different isotype classes of YF-specific antibodies. Interestingly, YF-specific IgM production seemed to start in young vaccinees slightly earlier than in old, whereas the IgG and IgA responses were indifferent between both age groups. Moreover, onset of detectable YF-specific IgM production showed a good correlation with neutralizing titers on day 10, collectively suggesting that the generation of early neutralizing capacity is probably mediated through IgM antibodies, as shown for smallpox vaccination (Moyron-Quiroz *et al.*, 2009) and is critically delayed in elderly vaccinees. Beside the aspect of direct virus neutralization, IgM antibodies have been shown to be highly efficient at opsonisation compared to IgG (Park and Nahm, 2011). Thus a small age-related delay in IgM production might be significant for virus elimination in old age. In addition, the produced IgM amount might be reduced in our aged cohort, but our semi-quantitative IIFA did not allow a precise comparison, though reduced IgM and IgA responses to pneumococcal booster vaccination have been reported for old people (Ademokun *et al.*, 2011; Park and Nahm, 2011). It remains open for future research, to which extent each isotype is contributing to the total neutralizing capacity and how much this is altered in the elderly. Another interesting aspect is antibody affinity and avidity, which has been shown to be decreased in primary responses in aged mice (Doria *et al.*, 1978). For humans, study results in this respect are ambiguous, with reports finding no age-related differences (Sasaki *et al.*, 2011; Stiasny *et al.*, 2012) or decreased affinities in the elderly (Khurana *et al.*, 2012). As antibody affinity has not yet been investigated in the context of a primary immune response against live-virus in

humans, a potential impact on virus elimination needs to be determined by in the future.

4.3 Increased acute plasmablast responses in the elderly

In addition to our analysis of serological immunity, we assessed the B-cell response, in particular acutely induced plasmablasts. Plasmablasts usually generated in the germinal center reaction are major producers of antibodies in the acute response phase and might differentiate into secondary lymphoid organ-resident plasma cells. We identified plasmablasts with the marker combination $CD19^{low}/CD20^{neg}/CD27^{hi}/CD38^{hi}$ (Odendahl *et al.*, 2005; Fink, 2012), but as we did not include CD138 we could not define the proportion of putative plasma cells within this population (Caraux *et al.*, 2010; Qian *et al.*, 2010). Our analysis revealed a distinct peak of acutely induced plasmablasts around day 10 and 14 post vaccination in both age groups, resembling a typical primary response kinetic, which has been previously reported by us and another group for YF vaccination and primary dengue virus infection (Kohler *et al.*, 2012; Wrammert *et al.*, 2012). This is in contrast to secondary responses e.g. to inactivated influenza and tetanus vaccinations, which show a transient acute plasmablast response around day 7 (Cox *et al.*, 1994; Odendahl *et al.*, 2005; Wrammert *et al.*, 2008; He *et al.*, 2011). Unexpectedly, the magnitude of the YF induced acute plasmablast response was significantly higher in our elderly cohort than in the young, which is in stark contrast to secondary flu vaccinations, where the opposite was found (Sasaki *et al.*, 2011; Frasca *et al.*, 2012). As we did not further characterize the plasmablasts, we can only speculate about the underlying cause for our observation. Thus it might be possible that (A) plasmablasts from aged individuals have a lower per-cell antibody production or express antibodies of lower affinity, requiring more plasmablasts to compensate this defect. However, in flu vaccination no such qualitative difference was found (Sasaki *et al.*, 2011). It might also be possible that (B) other specificities than against YF, so called bystander specificities, are induced more pronounced in the elderly. So far bystander induction has been investigated only in young adults and while bystander induction evidently takes place (Bernasconi *et al.*, 2002), the proportion of unrelated plasmablasts in the total plasmablast response strongly varies between different reports (Odendahl *et al.*, 2005; Wrammert *et al.*, 2008, 2012; Lee *et al.*, 2011; Hong *et al.*, 2013). Alternatively, the stronger plasmablast peak might reflect (C) an exaggerated immune response in the elderly, due to e.g. less efficient early virus control and thus prolonged presence of antigen. Indeed, in dengue infection the magnitude of plasmablast induction

correlates with disease severity (Garcia-Bates *et al.*, 2013). It should be noted that more plasmablasts lead to more virus-specific antibodies (Wrammert *et al.*, 2012), but not automatically to higher virus-neutralizing antibody titers as seen in our study and in dengue infections (Garcia-Bates *et al.*, 2013), rendering an exaggerated plasmablast response not necessarily more effective. Interestingly and analogous to the plasmablast response, we observed as well exaggerated YF-specific $CD4^+$ T-cell responses in our elderly vaccinees, as discussed later on. As both response magnitudes correlated with each other, it is very likely that both can be attributed to the same underlying cause, e.g. ineffective early virus control.

Apart from plasmablasts, we monitored naive and memory B cells in the course of the response. Both showed very distinct kinetics, which are probably specific migration patterns, as discussed in chapter 4.8. Probably due to our small study size we could not observe the modest age-related decline in naive B cells, reported in the literature (Johnson and Cambier, 2004). Further, pre-vaccination numbers of naive B cells were not predictive for subsequent plasmablast or neutralizing antibody responses, but there was the interesting finding that vaccinees with higher numbers of naive B cells on day 7 mounted subsequently stronger plasmablast responses. As day 7 marks the low point in the peripheral naive B-cell kinetic, which we interpret as a sign of migration to secondary lymphoid organs, it is imaginable that higher remnant numbers of naive B cells at this day reflect compromised lymphoid homing. In consequence, virus elimination and humoral response is possibly delayed, triggering a (compensatory) exaggerated plasmablast response. Although this is a highly speculative assumption, reports from aged mice indeed demonstrated reduced germinal center formation (Kosco *et al.*, 1989; Szakal *et al.*, 1990; Han *et al.*, 2003). Another study showed an altered composition of germinal centers in aged human tonsils (Kolar *et al.*, 2006). This and the fact that such a correlation was found only for naive but not for memory B cells on day 7 speak for decisive age-related alterations in the early phase of the naive B cell response, that need detailed analysis in future studies.

4.4 Diminished acute YF-specific $CD8^+$ T-cell responses in the elderly

$CD8^+$ T cells, capable of killing virus-infected cells, represent an important immune effector mechanism essentially limiting virus dissemination in the body. As recently demonstrated in the murine YF model, $CD8^+$ T cells complement humoral protection mediated through YF-neutralizing antibodies. In a B-cell knockout situation 25 % of mice survived an otherwise

100 % lethal intracerebral YFV injection (Bassi *et al.*, 2015). For tracking YF-specific $CD8^+$ T cell in the acute phase, we took advantage of the expression of the activation marker combination CD38 and HLA-DR, which can be measured directly *ex vivo* and which is applicable to all vaccinees regardless of their HLA types. In this kind of measurement it is unavoidable that some unspecific $CD8^+$ T cells are co-detected, as seen in our baseline data, however $CD8^+$ T cells expressing $CD38^+/HLADR^+$ in the acute response phase (between day 4 and 28) are almost exclusively YF-specific, as demonstrated in several publications (Miller *et al.*, 2008; Akondy *et al.*, 2009, 2015; Ahmed and Akondy, 2011; Kohler *et al.*, 2012; Blom *et al.*, 2013; DeWitt *et al.*, 2015). In accordance with these reports, YF-specific $CD38^+/HLADR^+$ $CD8^+$ T cells in our study expanded extensively until about day 17 and returned to basal levels by day 28, which we could demonstrate was kinetically true for both age groups. However, the magnitude of the YF-specific $CD8^+$ T cell response was strikingly reduced in the elderly, which is another key finding of our study and confirms for the first time similar observations made in various aged animal models (Murasko and Jiang, 2005; Brien *et al.*, 2009; Nikolich-Žugich *et al.*, 2012) for a human setting. Thus, both major anti-viral effector mechanisms, neutralizing antibodies and specific $CD8^+$ T cells, are obviously quantitatively diminished in the elderly, which we assume affects clearance of YF virus.

We furthermore investigated possible qualitative differences in the YF-specific $CD8^+$ T-cell response for which we stimulated PBMCs with the very immunodominant HLA-A0201-restricted peptide LLWNGPMAV (Akondy *et al.*, 2009). Only 5 young and 5 old vaccinees were carriers of HLA-A0201 in our study and thus statistics was underpowered. Nevertheless and in agreement with published data (Akondy *et al.*, 2009; Querec *et al.*, 2009), specific cell numbers in the acute phase obtained by peptide stimulation strongly overlapped with our CD38/HLADR measurements, indicating that results of the $HLA\text{-}A0201^+$ subgroup are probably applicable to all YF vaccinees. Interestingly, YF-specific $CD8^+$ T cells in aged vaccinees were characterized by increased expression of Granzyme A and B and decreased amounts of Perforin, while IFNγ, IL2 and CD40L expression was indifferent, which is in contrast to a publication comparing West-Nile-specific $CD8^+$ T cells in young and old infected individuals, reporting no qualitative differences at all (Lelic *et al.*, 2012). However, these West-Nile-specific $CD8^+$ T cells were measured 3-4 months after disease onset and therefore might not reflect the acute state we observed within the first month after infection. This time difference might also explain why this group could not find any age-difference in the West-Nile-specific $CD8^+$

response magnitude in an earlier publication (Parsons *et al.*, 2008). Whether the observed age-related qualitative alterations, which certainly require validation in larger cohorts, further deteriorate the already quantitatively reduced YF-specific $CD8^+$ T cell response, remains an open question. Future studies might employ *ex vivo* functional assays, e.g. specific killing assays of peptide-pulsed or infected target cells, which we could not conduct in this study due to the restricted blood volume we were limited to. Such analysis may help to uncover qualitative deficits in YF-specific $CD8^+$ T cells in the elderly.

Beside that, it would be interesting to analyze the influence of the individual HLA composition, which we, except for HLA-A0201, did not consider in our study. In acute dengue patients it has been shown that certain HLA alleles correlate with stronger $CD8^+$ response magnitudes (Weiskopf *et al.*, 2013). Therefore individual HLA composition might bias our quantitative results and should be included in future studies, though this will require much larger cohorts. Another aspect is bystander activation, which has been shown to exist in the $CD8^+$ compartment in severe infections, such as with Hepatitis B, dengue, adeno-, hanta- and Influenza A virus, where a significant proportion of $CD38^+$/HLA-DR^+ $CD8^+$ T cells are CMV or EBV specific (Tuuminen *et al.*, 2007; Sandalova *et al.*, 2010; Rivino *et al.*, 2015). But this has not been observed after experimental YF or smallpox infection (Miller *et al.*, 2008) or naturally-acquired TBE virus infection (Blom *et al.*, 2015). The reason for these conflicting observations might be due to the fact that severe virus infections cause high levels of inflammation, inducing frequencies of more than 20 % of acutely activated $CD38^+$/HLA-DR^+ $CD8^+$ T cells. In contrast, the infection with attenuated YF vaccine virus is usually very mild and typically induces $CD38^+$/HLA-DR^+ $CD8^+$ T-cell frequencies below 10 %. As we did not determine CMV or EBV specific $CD8^+$ T cells in the acute phase and such an analysis has never been done in aged donors, we cannot rule out that bystander CMV or EBV-specific $CD8^+$ T cells contributed to the measured $CD8^+$ response magnitude in our study. In case this had occurred in our elderly vaccinees, their net $CD8^+$ response would have been even lower, which thus would have even worsened virus elimination.

Future studies should also address $CD8^+$ T-cell clonality, which has been very recently investigated in YF vaccination in young adults. Based on deep sequencing of the CDR3 locus of the TCRβ chain, DeWitt *et al.* identified on average 2.000 YF-specific $CD8^+$ T-cell clones at the peak of the acute response in each subject (DeWitt *et al.*, 2015). It would be interesting, whether clonality is reduced in aged vaccinees, which could be another factor beside

quantitative and qualitative differences influencing vaccination outcome, though quite large amounts of blood are required for proper analysis.

Collectively, the cytotoxic $CD8^+$ T-cell response appeared to be considerably reduced in aged YF vaccinees, which might be detrimental in the anti-viral defense.

4.5 Quantitative and qualitative alterations in the acute YF-specific $CD4^+$ T cell response in the elderly

We next studied the YF-specific $CD4^+$ T-cell response, which has been analyzed so far only in young adults in a limited number of publications (Kohler *et al.*, 2012; Blom *et al.*, 2013; James *et al.*, 2013). We assessed YF-specific $CD4^+$ T cells in two ways: A) In analogy to the assessment of YF-specific $CD8^+$ T cells and similarly to Blom *et al*. (Blom *et al.*, 2013), we tracked them by the co-expression of Ki-67 and CD38. And B) we performed *ex vivo* short-term stimulations of blood with whole vaccine and detected YF-specific $CD4^+$ T cells by ICS. Both types of measurements showed a high degree of concordance in the acute phase in most of the vaccinees, though the stimulation assay showed higher variability. Both age groups started to mount comparable early acute Ki-67^+/$CD38^+$ $CD4^+$ T-cell responses. But interestingly, while the response in nearly all young adults was culminating already on day 10, in about half of the aged vaccinees $CD4^+$ T cells continued to expand until day 14, leading to a significantly enhanced total $CD4^+$ expansion in the aged group, which is consistent with data from aged macaques after primary WNV infection (Wertheimer *et al.*, 2010). Of note, good early YF-specific $CD4^+$ T-cell responses e.g. on days 4, 7 and 10, but not later, seemed to be key for better subsequent humoral and cytotoxic T-cell responses and in turn earlier viral control. In contrast, increased late expansion on day 14 was strongly associated with delayed virus control and, as outlined before, with the exaggerated plasmablast response. Thus it seems to us, that both increased late $CD4^+$ expansion and the inflated plasmablast response in the elderly are a consequence of the same cause, possibly prolonged presence of YF antigen due to delayed virus control.

Overall, we revealed on the one hand and as expected from textbook knowledge, a beneficial effect of quantitative early YF-specific $CD4^+$ responsiveness on anti-viral effector mechanisms, though an influence of age was not statistically detectable in this early phase. On the other hand, under certain circumstances, acute $CD4^+$ T-cell responses can become also prolonged or exaggerated, as seen in many aged YF vaccinees, which we think represents perhaps a countermeasure to still adequately fight back a viral attack in an immunosenescent situation

with a weakened virus defense. Interestingly, a study comparing $CD4^+$ T-cell responses in healthy adult and frail elderly after flu vaccination as well observed a positive association between early $CD4^+$ responsiveness and humoral immunity. They also could show an unfavorable prolonged specific $CD4^+$ T-cell response in frail subjects, though they report in contrast to us no excessive responses, which might be in their study due to the application of a non-replicating vaccine (Deng *et al.*, 2004). Nonetheless, also alternative explanations for the exaggerated response such as increased bystander activation in the elderly should be considered, though at least in young YF vaccinees Tetanus-toxoid-specific memory $CD4^+$ T cells were disproven to express the proliferation marker Ki-67 in the acute response phase (Kohler *et al.*, 2012), which, however, does not rule out bystander activation of other specificities with less stringed activation requirements such as EBV- or CMV-specific $CD4^+$ T cells.

Assessing YF-specific $CD4^+$ T cells by stimulation assay and ICS, revealed a typical anti-viral response (Swain *et al.*, 2012) dominated by T_H1 cells, expressing TNFα and IFNγ, as shown earlier by our group (Kohler *et al.*, 2012). Quantitative age differences in the cytokine production of TNFα, IFNγ and IL4 were found only on day 2 and 7, but no further correlations with anti-viral effector mechanisms could be identified. Beside the age aspect, we observed three other interesting findings: First, there is a small but significant peak of YF-specific $CD4^+$ T cells on day 2, preceding the main response, which becomes apparent after day 7. The same observation was made also by Bethke and Kohler (Bethke, 2011; Kohler *et al.*, 2012). It is possible that this early peak reflects priming or re-activation of $CD4^+$ T cells at the injection site, whereas the main response is probably a result of massive priming and expansion in lymphoid tissue, a notion supported also by our T-cell migration patterns, as discussed later. It would be interesting to verify, whether these early YF-specific T cells origin from truly naive or cross-reactive memory T cells (Welsh and Selin, 2002; Bacher *et al.*, 2013; Su and Davis, 2013), which could be approached by a deep TCR repertoire sequencing. A possible involvement of cross-reactive memory is supported by the fact that already prior vaccination all vaccinees possessed very small amounts of $CD4^+$ T cells, reactive to stimulation with the YF vaccine, despite rigorous exclusion of individuals with flavivirus-cross-reactive serology. Furthermore, these pre-vaccination cells expressed almost exclusively TNFα and/or IFNγ, but not e.g. IL2, rendering them very similar to the phenotype observed on day 2, but very different to YF-specific $CD4^+$ T cells of the main response, predominantly co-expressing IL2. In addition, pre-vaccination numbers of these cells also positively correlated with the early peak on day 2

(data not shown). Thus we think, YF-specific $CD4^+$ T cells on day 2 most likely descend from apparently cross-reactive $CD4^+$ T cells, though it is unclear to which extent they contribute to the subsequent main response and whether this has any effect on other aspects of the immune response and viral clearance.

Second, we observed IL4 being produced predominately by T_H1-like cells throughout the response, rather than by classical T_H2 cells, thus questioning the fixed T_H1/T_H2 paradigm. Our findings fit to early human *in vitro* studies reporting that IFNγ and IL4 expression are not mutually exclusive features and can be co-expressed (Maggi *et al.*, 1992). Also human memory T_H1 cells can acquire the capacity to additionally express IL4 *in vitro* (Messi *et al.*, 2003) and mixed T_H1/T_H2 phenotype cells expressing both master transcription factors GATA-3 and T-bet or the cytokines IFNγ and IL4 at the same time can arise *in vivo* in mice after primary infection with a parasite (Peine *et al.*, 2013; Deaton *et al.*, 2014). Our observations confirm the existence of such cells *in vivo* in human subjects in the context of a strong T_H1-inducing immune response, suggesting that T_H1 and T_H2 cell fates are more plastic than previously thought, although detailed further phenotypic and epigenetic analysis is needed. Moreover, it would be interesting to explore, whether those cells fully take over functions believed to be exerted by classical T_H2 cells and which advantage they possess by expressing both, T_H1 cytokines and IL4.

The third interesting observation was the transient occurrence of IL17/IL22-expressing YF-specific $CD4^+$ T cells. With an early peak on day 7, those cells displayed a kinetic, strongly differing from the general YF-specific $CD4^+$ response. Although a minor subset, we revealed that they were highly polyfunctional, always co-expressing IL2, TNFα and partially IFNγ, which is not unexpected given the high plasticity of T_H17 cells (Annunziato and Romagnani, 2009; Romagnani *et al.*, 2009; Cosmi *et al.*, 2014; Geginat *et al.*, 2014). So far, the role of T_H17 cells in fighting acute viral infections is ill defined, thought presence of virus-specific T_H17 cells have been documented e.g. for HIV, CMV and acute viral hepatitis (Yue *et al.*, 2008; Hou *et al.*, 2013). Since our study is the first to show the human T_H17 dynamics to an acute viral infection, it is difficult to define the exact role of these T_H17 cells in the YF-specific immune response. In view of the early peak of T_H17 cells on day 7, preceding the main $CD4^+$ response, one could speculate that they enter the site of inflammation earlier, thereby promoting subsequent recruitment of other immune cells, e.g. of T_H1 and cytotoxic $CD8^+$ T cells, in analogy to what has been observed in mice during Mycobacterium tuberculosis infection,

adenovirus-induced acute hepatitis and ovalbumin vaccination (Khader *et al.*, 2007; Pötzl *et al.*, 2008; Hou *et al.*, 2013). In this context one also should note the second IL17 peak on day 21, clearly succeeding the main $CD4^+$ response. It is possible that these late T_H17 cells, that might have acquired the capacity to express e.g. IL10 (Ayyoub *et al.*, 2009; Sallusto *et al.*, 2012; Geginat *et al.*, 2014), mediate in the contraction phase an immune-dampening function, but for a more definite conclusion a deeper phenotypic characterization of those cells at different timepoints, e.g. by mass cytometry, would be required.

Having assessed in parallel six different cytokines allowed us to determine not only quantity but also the composition of the YF-specific $CD4^+$ T-cell response. Changes in the functional composition of YF-specific $CD4^+$ T-cells were mostly observed over time rather than between both age groups. Thus it became apparent that the qualitative composition on day 7 was different from day 14, which differed again from day 28, reflecting possibly functional adaption required in different phases of the immune response. The time course however also revealed that in comparison to young vaccinees, particularly the monofunctional $IL2^+$ $CD4^+$ T-cell subset, lacking other investigated effector cytokines, was strongly inflated around the $CD4^+$ response peak in the elderly. This would fit well to the increased expansion of Ki-67^+/$CD38^+$ $CD4^+$ T cells in aged vaccinees and indeed we identified a highly significant correlation between those IL2 single-producers e.g. at day 14 and the acutely-induced $CD4^+$ expansion. Whether this particular subset is a response to the deferred viral clearance or just a driving force to enhance proliferation in elderly YF vaccinees is difficult to tell, however the fact that it represents a very prominent subset constituting up to a quarter of the total YF-specific response on day 14 and 17 in the elderly suggests an important role.

Related to this is another cardinal finding of this study. YF-specific $CD4^+$ T cells in elderly vaccinees were significantly less polyfunctional and conversely more monofunctional than their counterparts in young individuals. Although summing up cytokine expression seems to be a rather arbitrary measure, increased ability of a particular cell to co-express several cytokines has been shown to be associated with higher cytokine expression levels and better other functionalities (Darrah *et al.*, 2007; Kannanganat *et al.*, 2007), which we observed in our study as well (data not shown). Accordingly, the qualitative polyfunctionality index has been reported to relate to improved pathogen control and better vaccination responses (Seder *et al.*, 2008). Strikingly, also in our study, higher $CD4^+$ polyfunctionality, characteristic for young vaccinees, was strongly correlated to better neutralizing antibody titers and $CD8^+$ T-cell

responses. Thereby, our study proves that age clearly impacts qualitative functionality in an acute primary response.

Altogether, we uncovered age-related alterations in the YF-specific $CD4^+$ T-cell immunity at the quantitative and qualitative level and both seemed to be interlinked with each other, as specific $CD4^+$ T-cell numbers at day 10 correlated with the capacity to co-express multiple cytokines. Our correlation analysis further revealed that these alterations likely affect humoral and cellular effector responses and thereby also viral clearance.

4.6 Impact of pre-vaccination T-cell composition, in particular of naive $CD8^+$ T cells and $CD4^+$ RTE, on the immune response to YF

Having identified prominent age-related alterations in YF-specific adaptive immunity, we next sought to find immune signatures prevalent before or early after vaccination that critically influence subsequent adaptive immunity and vaccination outcome. Several studies have described age-related, compositional changes in circulating T-cell phenotypes at the steady state (Hong *et al.*, 2004; Saule *et al.*, 2006; Weinberger *et al.*, 2007; Provinciali *et al.*, 2009; Di Benedetto *et al.*, 2015) and some have also shown an effect on booster vaccinations (Trzonkowski *et al.*, 2003; Kang *et al.*, 2004; Moro-García *et al.*, 2012; Derhovanessian *et al.*, 2013). However, whether and how the present phenotypic T-cell composition influences a *de novo* immune response has not been explored so far in humans, which led us to investigate this subject with a special focus on naive T cells, assumed to play a pivotal role in primary responses. Our immunophenotyping at day 0 revealed a significant reduction of naive $CD8^+$ T cell numbers in the elderly, which has been reported to be a very prominent compositional change with age (Fagnoni *et al.*, 2000; Appay and Sauce, 2014). Differences in other T-cell subsets, such as EM and Eff T cells did not reach statistical significance between both age groups, though studies with larger cohorts were able to reveal differences in some of those individually more variable T-cell subsets (Fülöp *et al.*, 2013; Adriaensen *et al.*, 2015; Di Benedetto *et al.*, 2015). Interestingly, although numbers of naive $CD4^+$ T cells were comparable between young and old subjects, confirming a recent cross-sectional study (Wertheimer *et al.*, 2014), the frequency (and in consequence also number) of CD31-expressing $CD4^+$ RTE, contained therein, was highly significantly reduced in aged individuals in our study. This is a clear sign of reduced thymic activity in the elderly and was expected from a number of earlier studies (Kimmig *et al.*, 2002; Gomez *et al.*, 2003; Kohler *et al.*, 2005; Junge *et al.*, 2007; Kilpatrick *et al.*, 2008; Tanaskovic *et al.*, 2010; den Braber *et al.*, 2012). While we

did not analyze the naive TCR repertoire directly, our $CD4^+$ RTE data strongly implies that our aged study participants had a less diverse TCR repertoire than the young ones, as $CD4^+$ RTE have been demonstrated to be the primary, essential repository for highly diverse naive $CD4^+$ T cells comprising also newly generated T-cell specificities (Kohler *et al.*, 2005).

But do reduced pre-vaccine numbers of naive $CD8^+$ T cells and $CD4^+$ RTE, characteristic for the elderly vaccinees, matter when it comes to a primary infection? The correlation analysis we performed suggests, that indeed weaker YF-specific T-cell responses are strongly associated with limited availability of naive $CD8^+$ T cells and $CD4^+$ RTE prior to vaccination. For example, vaccinees with low amounts of $CD4^+$ RTE clearly mounted quantitatively reduced, early YF-specific $CD4^+$ T-cell responses and generated less polyfunctional YF-specific $CD4^+$ T cells. This key finding of our study, experimentally demonstrated for the first time in humans, was not completely unexpected as experiments in mice have suggested this association (Yager *et al.*, 2008; Blackman and Woodland, 2011). But given the fundamental difference in naive $CD4^+$ T-cell homeostasis between mice and man, which relies in man predominately on peripheral proliferation rather than on thymic output (den Braber *et al.*, 2012), it is remarkable that antigen-specific adaptive T-cell immunity after primary infection is nevertheless strongly determined by the number of $CD4^+$ RTE and not by total naive $CD4^+$ T cells. This points out that thymic output, as the only process to generate new T-cell specificities, is of essential importance for the generation of an effective primary response to a neoantigen. Notably and in opposite to what has been found in booster vaccinations, the pre-immune status of memory or effector T cells did not influence the YF-specific response, emphasizing again the predominant role of naive $CD4^+$ RTE in a *de novo* challenge.

For $CD8^+$ T cells we could show that individuals with low initial numbers of naive $CD8^+$ T cells generate subsequently weak YF-specific $CD8^+$ T-cell responses. Obviously here the size of the total naive $CD8^+$ T cell pool is already a decisive factor, similar to what has been reported from primary infections in rhesus monkeys (Cicin-Sain *et al.*, 2010). For yet unknown reasons, thymic involution affects the naive $CD8^+$ T-cell compartment much more than its entire naive $CD4^+$ counterpart (Appay and Sauce, 2014). In consequence, age-related changes, such as contraction of the naive pool and loss of the naive repertoire diversity (Buchholz *et al.*, 2011), become apparent earlier and more noticeable in naive $CD8^+$ T cells than in naive $CD4^+$ T cells. The initial number of total naive $CD8^+$ T cells in our study is hence strongly connected to individual thymic activity in a similar way as $CD4^+$ RTEs. Both parameters also correlate highly

significantly with each other (data not shown). Thus in analogy to $CD4^+$ RTE, limited availability of naive $CD8^+$ T cells is a factor that critically dictates the magnitude of a primary YF-specific $CD8^+$ T-cell response.

Our observations provide a plausible explanation for the high incidence of YF-SAE in thymectomized vaccinees (Eidex, 2004), who rely for obvious reasons solely on peripheral naive T-cell proliferation and cannot produce new T-cell specificities. Similarly, reduced numbers of $CD4^+$ RTE and naive $CD8^+$ T cells might be a reason for the increased incidence of YF-SAE in individuals older than 60 (Monath *et al.*, 2005; Rafferty *et al.*, 2013). Although only some study participants were older than 60 and none of the vaccinees experienced YF-SAE, we might have observed mild first signs of a beginning diminution of responsiveness due to reduced thymic output and repertoire loss in our old study cohort. At e.g. more advanced age, the accumulated lack of many essential T-cell specificities, in other words the increased presence of "holes" in the TCR repertoire (Yager *et al.*, 2008), found also in studies with aged mice (Smithey *et al.*, 2012), might prevent under certain circumstances the generation of a sufficient primary response repressing spread of the YF vaccine virus. Alternatively or in addition to complete extinction of certain naive TCR specificities, which are estimated at least to range between 100 and 1000 T cells per clone in humans (Arstila *et al.*, 1999), it is imaginable that at advanced age individual clone size is quantitatively reduced to such an extent that secondary lymphoid organs are insufficiently covered (Buchholz *et al.*, 2011). Thus, the few remaining naive T cells might reach the site of infection too late and/or are insufficiently primed. It would be highly interesting to elucidate directly whether naive TCR repertoire constriction or clone size reduction or a combination of both is responsible for the reduced responsiveness to *de novo* challenges in the elderly. Thus, for future confirmatory studies it would be indispensable to include a detailed TCR repertoire analysis, e.g. by a deep sequencing approach. By a highly sophisticated combination of Tetramer-enrichment of naive $CD8^+$ T cells (Alanio *et al.*, 2010) and deep-sequencing it would be even possible to assess and compare quantity and quality of certain YF-epitope-specific naive precursors between young and old vaccinees. Another aspect we have not touched is to test naive functionality *in vitro* and relate it to *in vivo* correlations. Altogether, reduced availability of naive CD8 T cells and $CD4^+$ RTE in the elderly is an immune signature associated with poor primary responsiveness to YF vaccination and with further validation both measures could be included in pre-vaccination risk assessment strategies for elderly or immune compromised individuals.

4.7 Age-alterations in YF-induced innate immunity and the impact on adaptive immunity

Innate immunity provides the basis for efficient initiation of an adaptive immune response. It has been shown that YF vaccination strongly induces an innate immune response in the first days after vaccination (Gaucher *et al.*, 2008; Querec *et al.*, 2009; Kyogoku *et al.*, 2013). However, it is completely unknown, which and how different innate cell types are induced and whether aging affects the innate response to experimental YF infection. Our tight immunophenotyping of peripheral innate cell types revealed distinct kinetic patterns: Some innate subsets such as pDCs and $CD11c^+$ mDCs showed a sharp early peak at day 4, while others such as $CD14^+/CD16^+$ nonclassical monocytes peaked slightly later at day 7. Finally, several subsets such as $CD141^+$ mDCs and basophils did not show such early peaks and remained stable or transiently declined in numbers. Thus, YF vaccination seems to trigger an orchestrated innate sequence of distinct quantitative changes in blood, which likely reflects migration patterns to or from secondary lymphoid organs or the site of infection. Although the observed early dynamics in innate immunity after YF infection did not contain very prominent age signatures, they are from an immunological point of view highly interesting and have not yet been reported in humans, as only experimental infections provide the opportunity to study such early time points after inoculation in humans. At least for a better understanding of the YF-induced pDC dynamics, we can refer to work done in rhesus macaques infected acutely with Simian immunodeficiency virus (SIV) (Malleret *et al.*, 2008a; Brown *et al.*, 2009; Bruel *et al.*, 2014). Comparable to our data, these studies report an acute pDC peak between days 3-6 post SIV infection, followed by a strong decline below baseline numbers by day 10. This concerted disappearance from the periphery could be explained by migration to lymph nodes (LN) as blood-derived pDCs started to accumulate and produce IFNα there. Yet, the origin of the early pDC peak remains unclear from the SIV studies, which only could show that at this stage pDCs were not activated, proliferating nor acutely mobilized from bone marrow. Thus, the early pDC peak can only be explained by a transient mobilization of pDCs from peripheral and or secondary lymphoid tissue into the blood stream, though this remains to be proven. The information on pDCs can be to some extent probably also applied to other innate subsets, such as recently reported for $CD14^+/CD16^+$ monocytes (Kwissa *et al.*, 2014), but for a complete understanding of the observed patterns an even deeper immunophenotyping, such as by mass cytometry, needs to be done, which could include analysis for chemokine receptors, activation markers and proliferation state.

As already mentioned, differences related to age were less prominent in the early innate dynamics. Rather, we observed striking quantitative age differences in certain innate subsets, such as pDCs, $CD11c^+$ mDCs and $CD14^+/CD16^+$ nonclassical monocytes, which became even more apparent after onset of the innate immune response. Thus, most of the elderly are clearly compromised in their pDC and mDC compartments, confirming several reports obtained at the steady state (Shodell and Siegal, 2002; Pérez-Cabezas *et al.*, 2007; Jing *et al.*, 2009; Garbe *et al.*, 2012; Orsini *et al.*, 2012; Verschoor *et al.*, 2014a). Further about half of them showed a monocyte profile skewed towards pro-inflammatory nonclassical monocytes (Sadeghi *et al.*, 1999; Seidler *et al.*, 2010; Hearps *et al.*, 2012; Verschoor *et al.*, 2014b), thought to be associated with "inflamm-aging". These signatures become also major determinants when peripheral innate immunity is viewed in its entirety by multivariate analysis such as PCA or clustering. Better than each innate immune parameter alone, both systemic approaches segregated well old from young vaccinees based upon their complete individual innate immune states at day 4, indicating the existence of age-typical innate immune conditions. However, age-segregation was not always perfect. For example, three young individuals had relatively low pDC and high nonclassical monocytes counts, characteristic of an aged innate immune system and thereby were grouped to old vaccinees. Vice versa, one old person exhibited features of a well preserved innate immune system and consequently was clustering with young individuals. Thus, aging strongly affects innate immunity, but there is additional individual variability beyond the level of pure age-discrimination, probably attributed to other in- and/or extrinsic factors.

Mild adverse reactions after YF vaccination, such as fever, headache or myalgias, not to be confused with YF-SAE, fall within the time window of innate responsiveness and are thought to be caused by the release of systemically-effective cytokines such as TNFα, IL1α and IFNα (Barnett, 2007). From two large studies it has been reported that elderly YF vaccinees have a reduced incidence of these mild reactions (Monath *et al.*, 2002, 2005), which was observed also in our study. Our results indicate, that this reduced incidence of mild reactions may be attributed to low numbers of pDCs, producing pro-inflammatory cytokines (Hacker *et al.*, 1998), as those YF vaccinees in our study without any symptoms after vaccination, i.e. mostly elderly ones, had significantly fewer pDCs. In addition, but not explored by our study, further functional age-related defects at the cellular level might apply (Panda *et al.*, 2010; Agrawal and Gupta, 2011; Garbe *et al.*, 2012; Solana *et al.*, 2012; Qian *et al.*, 2015). Collectively, it

seems to us that absence of any mild symptoms along with low pDC numbers reflects a state of poor innate immune activation, which leads to the question; do those mostly age-related quantitative changes in innate immunity affect YF-specific adaptive immunity?

Our correlation analysis suggests that at least numbers of pDCs and $CD11c^+$ mDCs have a strong influence on the induction of adaptive immunity, i.e. low numbers of pDCs at day 4 were strongly associated with poor YF-specific $CD8^+$ responsiveness and reduced YF-specific $CD4^+$ polyfunctionality. Similarly, individuals with low $CD11c^+$ mDC counts developed weaker YF-specific $CD8^+$ and $CD4^+$ T-cell responses. Consistently, this could be visualized also in our multivariate-analyses. Although these associations were somewhat expected from textbook knowledge based mostly on animal experiments (i.e. strong induction of innate immunity is a pre-requisite for good adaptive responsiveness) our report is one of the few experimentally showing that this is indeed true also in humans being infected by a virus. Our data further points out the crucial role of DCs in YF vaccination, which have been in shown *in vitro* to become activated after sensing YF virus and to produce IFNα (Querec *et al.*, 2006; Mandl *et al.*, 2011; Bruni *et al.*, 2015). Consequently, our findings suggest that quantitative DC immunosenescence is, beside limited availability of naive $CD8^+$ T cells and $CD4^+$ RTE, a main factor for reduced adaptive responsiveness to YF vaccination in the elderly. This might be further restricted by age-related functional impairments in DCs, reported to occur at least in infections with other flaviviruses, such as WNV (Kong *et al.*, 2008; Qian *et al.*, 2011). To further validate our findings for pDCs, serum samples from our study are currently investigated for IFNα levels, which we expect to be decreased in the elderly and to correlate with adaptive immunity as suggested by observations in WNV-infected patients (Tobler *et al.*, 2008).

We did not observe significant correlations of adaptive responsiveness with innate cell types other than pDCs and $CD11c^+$ mDCs, although this cannot be ruled out due to the low statistical power of our study. For example, a recent publication comparing immune responses in African and European YF vaccinees found a strong association between high numbers of pro-inflammatory $CD14^+/CD16^+$ monocytes and poor humoral adaptive responsiveness (Muyanja *et al.*, 2014). There, the African cohort showed an elevated baseline level of $CD14^+/CD16^+$ monocytes, probably due to more frequent exposure to infectious diseases. It is possible that this is true also for aged vaccinees, as several but not all old participants in our study had increased numbers of pro-inflammatory $CD14^+/CD16^+$ monocytes as well. However, the low sample size did not allow us to investigate this effect with stratified data. Another

study that compared baseline immunological profiles of individuals with asymptomatic versus severe WNV infection identified high levels of $CD14^+/CD16^+$ monocytes and low levels of pDCs to be associated with a disease severity (Qian *et al.*, 2015). As age was roughly comparable between severe and asymptomatic patients it emphasizes the importance of the individual innate immune constitution, which is obviously not only shaped by age but also by other factors such as history of exposure to infectious diseases and which must be considered when evaluating immunological responsiveness. In this view it is interesting to note that those four individuals being "age-misclassified" in our multivariate analyses often exhibited adaptive responses closely resembling those of circumjacent subjects. For example, the three young vaccinees having low pDC and high $CD14^+/CD16^+$ monocyte counts, also did not generate very magnificent YF-specific $CD8^+$ T-cell responses, while one old vaccinee with a well preserved innate immune system, had a very strong $CD8^+$ response.

Further it is important to note that our correlation analysis cannot be used to infer causality. Although it is very likely that quantitative scarcity of pDCs and $CD11^+$ mDCs in the elderly leads to a state of poor innate immune activation and reduced antigen-presentation consequently affecting priming and actuation of adaptive immunity, alternative explanations might be possible. With DCs being a main target of infection by the virus and an important vehicle for virus replication and spread (Barba-Spaeth *et al.*, 2005; Palmer *et al.*, 2007; Gandini *et al.*, 2011), one can imagine that limited availability of these target cells in the elderly also alters virus propagation in the host. Thus it might be possible that virus spread occurs less quickly in the elderly, leading to a delayed sensing by and reduced induction of adaptive immunity, which would fit as well to our data set. Thus the exact causality for the observed correlations remains open and should be studied in future in animal experiments, e.g. with traceable YF virus. Furthermore, future YF studies might dissect the dependency of the three variables age, DCs and naive T cells of each other and the contribution of each to the observed effect on adaptive immunity. In other words it would be important to examine confounding and effect modification, which could be done by stratification or regression model analysis, though these require a much larger sample size than available in our study. Taken together our study revealed profound age-related alterations in innate immunity, especially in the DC compartment, which were associated with impairments in the acutely-induced YF-specific T-cell response. After further validations, DC numbers might thus be another immune signature that seems worthwhile to be considered in individualized pre-vaccination risk assessment.

4.8 Patterns of immune cell dynamics after YF vaccination and the possible influence on vaccination outcome

Direct enumeration of immune cells in peripheral blood at tight intervals enabled us to examine the temporospatial distribution and composition in the entire course of the infection. As indicated already before, we found very specific kinetic patterns. For example many innate cell types, such as monocytes and DCs showed an early peak around day 4 or day 7, as discussed in the previous chapter. Conversely, numbers of peripheral T and B cells significantly dropped from day 0 to day 7 in all vaccinees regardless of age and then started to reappear again, as also reported in a previous YF-study conducted by our group (Bethke, 2011). The same early transient drop in T-cell counts was observed after experimental primary inoculation of healthy adults with vaccinia virus (Zaunders *et al.*, 2006), which was accompanied by local LN tenderness around day 7. By day 10 after vaccinia infection, T-cell numbers increased again and LN swelling decreased, while inflammation and induration started at the inoculation site. This suggests that T lymphocytes from the periphery start to migrate massively to LNs, where they are sequestered around day 7 and probably meet antigen-presenting cells, such as pDCs or mDCs, which is also supported by data from primary SIV infection in macaques (Malleret *et al.*, 2008b; Bruel *et al.*, 2014). The subsequent increase of counts in the periphery reflects in part most likely the release of highly proliferating antigen-specific T cells, which indeed show the strongest increase in whole blood counts after day 7 in our study. Additionally and probably to a much greater extent, unrelated T-cell specificities attracted to the LN but not participating in the response might get released back to the blood stream. The concept that T and B lymphocytes massively home to secondary lymphoid tissue in the first 7 days after infection was further supported when we separately examined different cellular subsets. Indeed, only subsets with the capability to home to LNs, i.e. naive and CM T cells and naive and memory B cells which express CCR7 (Henneken *et al.*, 2005; Payne *et al.*, 2009) showed the characteristic drop towards day 7. Surprisingly also $CD56^{br}$ NK cells, known to express CCR7 (Campbell *et al.*, 2001), followed this pattern. Accordingly, subsets lacking CCR7 expression such as EM and Eff T cells and also $CD56^{dim}$ NK cells did not drop in comparison to baseline. The unexpected migration of both naive and CM T cells to LNs even in a primary response with no pre-existing memory nevertheless is reasonable, as the immune system does not know at this early stage whether it has already experienced the pathogen or not. As the subset-specific cell migration patterns extensively

correlated with each other within each individual, we hypothesized that they are dependent on the strength of innate immune activation; however neither counts of any DC nor monocyte subset correlated with the observed transient declines. Thus it remains open which factors induce this early migration wave to secondary lymphoid tissues, which probably depends on the CCR7-ligands CCL19 and CCL21 (Förster *et al.*, 2008) as shown in SIV infected macaques (Choi *et al.*, 2003). Currently investigated candidates are "acute response" cytokines such as IL1β or TNFα that can be released by epithelial cells or innate components after YF infection (Gaucher *et al.*, 2008; Pulendran, 2009; Querec *et al.*, 2009). Our other finding that the decline is strongly influenced by the initial cell number of the respective subset speaks for a stochastic migration. Based on this data and perhaps new data from the ongoing serum cytokine analysis a detailed modeling of the kinetic patterns seems to be appealing. But do these early migration dynamics affect the YF-specific adaptive response? In our correlation analysis we could not find any relationship, i.e. also old vaccinees, with diminished adaptive immunity showed migration patterns comparable to young vaccinees. Collectively, although larger studies might find a relationship, early B and T-lymphocyte migration seemed not to be a major factor influencing adaptive responsiveness.

4.9 Influence of CMV and EBV status on vaccination outcome

Chronic viral infections such as CMV and EBV infections are thought to accelerate immunological aging as they constantly challenge the immune system of their host (Brunner *et al.*, 2011). Therefore they represent potential confounders, which have to be considered when evaluating vaccination responsiveness. We analyzed the CMV/EBV baseline status in our vaccinees at the cellular and humoral level, which showed a high degree of concordance.

Our study was too small to reveal neither any age-related increase in CMV seropositivity shown in large population surveys (Staras *et al.*, 2006; Bate *et al.*, 2010) nor an increase of CMV-specific humoral and cellular immunity by age (Musiani *et al.*, 1988; Weymouth *et al.*, 1990; Khan *et al.*, 2004; Stowe *et al.*, 2007). As all participants were seropositive for EBV, we could only compare levels of EBV-specific humoral and cellular immunity, which has been reported to increase with age (Glaser *et al.*, 1985; Stowe *et al.*, 2007). However, similar to CMV, no age-difference was found between both age cohorts of our study. Nevertheless, CMV and EBV baseline status had a clear impact on distribution and phenotype of certain leukocyte subsets. For example, CMV seropositivity was strongly associated with a skewed monocyte to

mDC ratio. As CMV latently resides in monocytes (Sinclair and Reeves, 2014) and becomes activated upon differentiation e.g. into both mDC subsets (Reeves and Sinclair, 2013), it seems reasonable that that CMV infection alters monocyte differentiation and thus also the ratio in peripheral blood. Indeed, *in vitro* experiments have demonstrated that CMV can inhibit differentiation of monocytes to mature mDCs through a multitude of mechanisms (Gredmark and Söderberg-Nauclér, 2003; Gredmark-Russ and Söderberg-Nauclér, 2012), including expression of viral-encoded IL10 (Chang *et al.*, 2004; Raftery *et al.*, 2004; Avdic *et al.*, 2011). In analogy to that, it is possible that EBV affects also monocyte differentiation which might explain the inverse association of EBV immunity and mDC baseline counts observed in our study. In fact, studies have shown that EBV infects or enters monocytes and DCs (Savard *et al.*, 2000; Tugizov *et al.*, 2007) and prevents *in vitro* the development of DCs (Li *et al.*, 2002; Guerreiro-Cacais *et al.*, 2004). Regarding baseline T-cell constitution, we could show that CMV seropositivity led to increased numbers of Eff $CD8^+$ and EM $CD4^+$ T cells, which is consistent with previous reports (Chidrawar *et al.*, 2009; Litjens *et al.*, 2011; Wertheimer *et al.*, 2014). Also EBV-specific immunity seemed to drive Eff $CD8^+$ T-cell expansion in our study perhaps at the expense of CM cells, however no proper study exists that explored the impact of EBV on the phenotypic distribution of T cells. Importantly and consistent with other reports in man (Litjens *et al.*, 2011; Vescovini *et al.*, 2014; Wertheimer *et al.*, 2014) and mice (Mekker *et al.*, 2012; Smithey *et al.*, 2012), neither CMV nor EBV showed any association with reduced initial naive T-cell counts or $CD4^+$ RTE, suggesting that the observed quantitative decline is mostly attributable to age rather than to persistent infections. In this context and despite the CMV/EBV-induced alterations in innate and adaptive immunity, CMV or EBV status did not show any measurable influence on the YF-specific immune response, indicating that CMV/EBV driven alterations do not limit the ability of the host to respond to a novel infection, which is consistent with the reports of Lelic *et al*. and Vezys *et al*. (Vezys *et al.*, 2009; Lelic *et al.*, 2012). Nevertheless one should note, that given the size of our study, we cannot completely rule out any influence of persistent viral infections on the vaccine response outcome. Other studies in aged mice have reported a negative effect (Cicin-Sain *et al.*, 2012; Mekker *et al.*, 2012; Smithey *et al.*, 2012), but also a positive effect, especially in young CMV^+ individuals might be possible (Furman *et al.*, 2015). Collectively, persistent CMV and EBV infection clearly shape the immune system of their hosts, however their impact on an acute *de novo* challenge seem to be rather low in comparison to other immune signatures we have found.

4.10 Long-term YF-specific immunity in young and old vaccinees

YF vaccination is one of the most potent vaccines, conferring essentially life-long protection (Monath and Vasconcelos, 2015). Whether and how immune aging affects such long-term immunity has not yet been studied in great detail. We therefore re-invited our participants and assessed YF-specific humoral and adaptive immunity up to 3 years after primary vaccination. All YF vaccinees still demonstrated protective YF-neutralizing titers and no age-differences were found in titer levels or in their decline from day 28 levels, which has been observed also in a large YF vaccination trial (Monath *et al.*, 2005). In contrast, specific antibody levels induced by inactivated TBE and tetanus vaccinations have been shown to be age-dependent (Hainz *et al.*, 2005). Thus, YF live-viral vaccination seems to be potent enough to overcome effects of immune senescence on long-term humoral immunity otherwise relevant in e.g. inactivated vaccines. Similar to humoral immunity and despite the strong contraction of these cells from day 28 levels, all HLA-A0201$^+$ vaccinees still possessed detectable YF-specific CD8$^+$ T cells 2-3 years after vaccination. Consistent to acute WNV infection in humans (Lelic *et al.*, 2012), we did not observe any age-difference, though one should note that we only analyzed nine vaccinees. In contrast to this, long-term YF-specific CD4$^+$ T-cell immunity differed between young and old vaccinees. While numbers of YF-specific IL2, IL4 and TNFα producers were not significantly affected by age, CD4$^+$ IFNγ$^+$ T cells were clearly reduced in the elderly, which is also the underlying reason for the reduced frequency of polyfunctional CD4$^+$ T cells in the aged cohort. The consequence of these alterations in long-term memory in aged vaccinees is unclear, especially as there is no other comparable data available. In light of a recent WHO announcement suggesting that YF booster vaccinations are dispensable (Who, 2015), the altered YF-specific CD4$^+$ T-cell memory in our elderly vaccinees raises however the possibility that this recommendation may not be generally applicable to all elderly subjects.

Integrating pre-vaccination, acute response phase and long-term memory data, our study revealed that the magnitude of the acute YF-specific T-cell response strongly determines late-memory T-cell immunity, as predicted from fundamental work in LCMV-infected mice (Murali-Krishna *et al.*, 1998). Consequently, pre-vaccination CD4$^+$ RTE and early pDCs numbers, important for strong acute T-cell responses, also were closely associated with long-term YF-specific T-cell immunity, which emphasized that these initial and early immune signatures are as well key factors for the induction of proficient and long-lasting T-cell memory.

In addition, we also assessed which factors possibly influence the contraction of YF-specific immunity from day 28 to long-term levels. Interestingly, persistent CMV and EBV infection seemed to play a central role, as high levels of CMV-specific $CD4^+$ T cells were associated with a more profound contraction of YF-specific $CD8^+$ T cells, which is to some extent similar to what has been observed for EBV-specific $CD8^+$ T cells in the context of CMV seropositivity (Khan *et al.*, 2004). On the other hand and somewhat unexpected, strong EBV-specific immunity seemed to promote stability of YF-specific humoral immunity. Interestingly, humoral EBV immunity was also positively associated with CMV antibody levels in CMV^+ individuals in our YF study and in a flu-vaccination study we have conducted, which hints for a yet undefined role of EBV in the maintenance of humoral immunity, though this clearly requires validation in further studies.

Altogether our study provides evidence that long-term YF immunity, despite some qualitative defects in $CD4^+$ T cells, is relatively comparable in old and young vaccinees and largely dependent on the strength of the previous acute YF response. We hope that we can follow up our study cohorts also in the next years, in order to study whether age-related defects in YF-specific memory become perhaps apparent after a longer time.

4.11 Implications for YF vaccination in the elderly

The findings of our study might have several implications for YF vaccination in elderly people or immunocompromised subjects in general. At first, our humoral and cellular data together with the report by Roukens *et al*. (Roukens *et al.*, 2011), speaks for a slightly delayed onset of protection in elderly vaccinees. Thus it might be advisable to extent the recommended time window of 10 days before entering a YF endemic area for aged vaccinees.

At second and as indicated in the previous chapter, long-term immunity might be slightly altered in the elderly, raising the possibility that YF booster vaccinations are still required in certain risk groups. Although it has been reported that protective neutralizing antibody titers are maintained in elderly vaccinees that have been vaccinated more than 10 years ago (Coulange Bodilis *et al.*, 2011), which led to the conclusion that YF booster vaccinations are dispensable as outlined in a recent meta-analysis (Gotuzzo *et al.*, 2013), it is still possible that cellular immunity is not as long lasting in the elderly. Given that it is only assumed that humoral immunity alone gives complete protection (Slifka, 2014) and $CD8^+$ T cells complemented antibody-mediated protection in the murine YF model (Bassi *et al.*, 2015), the

decline in cellular adaptive immunity needs further investigation particularly in the aged. Thus, it might be advisable that for safety reasons YF booster vaccinations should be still applied in elderly vaccinees, especially as they are very well tolerated even at advanced age (Monath, 2012).

At third, persons at special risk to develop YF-SAE might benefit from an individual pre-vaccination risk assessment based on immune markers found in our study, such as numbers of $CD4^+$ RTE, naive $CD8^+$ T cells or pDCs. Although clearly these parameters need further validation and risk levels have to be established, the growing number of travelers exceeding the age of 60 could clearly benefit from it, as in many countries they otherwise would not be vaccinated under current recommendation guidelines. Further, this would improve safety in mass vaccination campaigns for aged or otherwise immunocompromised persons living in YF-endemic regions.

All this points also to future directions of vaccinology, in which many flavivirus-caused diseases, such as Dengue fever or Japanese encephalitis (JE) could be prevented by chimeric, live-viral vaccines based on a recombinant YF backbone (Bonaldo *et al.*, 2014). These vaccines are currently tested in phase 2/3 trials and commercial production, e.g. of ChimeriVax-JE will start soon (Monath *et al.*, 2015). In principle, all our findings can be translated to these new recombinant vaccines, which emphasize the importance of such investigations as millions of people including elderly will receive them. In this regard, it should be mentioned, that YFV-17D and YF-based live-viral vaccines are probably much more potent than any inactivated vaccine, especially in old people which often have difficulties to achieve protective immunity. On the other hand, vaccine safety needs to be rigorously assured in live-viral vaccinations, which again speaks for an individual pre-vaccination risk-benefit analysis supported by immunological signatures, such as e.g. $CD4^+$ RTE. Alternatively, an inactivated YF vaccine (Monath *et al.*, 2011) could be applied to such risk groups, which showed an efficacy and safety profile comparable to other inactivated flavivirus vaccines (Heinz and Stiasny, 2012). However, it is unclear, to which extent long-lasting protection is achieved with this vaccine in elderly persons (Hayes, 2010), which stresses the necessity of a comparative study between live-viral and inactivated YF vaccination for this age group.

4.12 Concluding remarks

Collectively, this study demonstrates that the primary immune response to a living virus is compromised in elderly humans on manifold levels, with naive T cells and DCs representing the most restrictive elements. It is widely accepted that these two cell types are limited in their availability in the elderly and therefore it is plausible that this contributes to the generally high susceptibility of the elderly to infections by newly emerging pathogens, such as WNV (Jean *et al.*, 2007) or SARS coronavirus (Peiris *et al.*, 2003). Importantly, age-related immune deficiencies in response to vaccination start to develop already in the early fifties, as seen in the relatively "young" aged cohort. Although these changes were relatively mild at that age, causing an only slightly prolonged YF viremia, they might become dangerous when extrapolated to individuals more advanced in age, which potentially explains why the incidence of life-threatening YF-SAE increases with age. Nevertheless, more immunological research should be done on samples of aged YF-SAE patients to definitely identify the cause of this severe complication.

This study further proves that albeit the response to YF vaccination was reduced in the elderly cohort, protective immunity was eventually achieved in all vaccinees regardless of age, which render YF-based vaccination strategies a powerful tool for immune specificities difficult to induce in aged individuals (Liniger *et al.*, 2007). In addition, this work points out that YF vaccination is an ideal model for basic immunologists to precisely investigate immune responses in humans. In this respect, the novel findings in aged YF vaccinees provide the basis for future studies uncovering the mechanisms of immune aging in the elderly.

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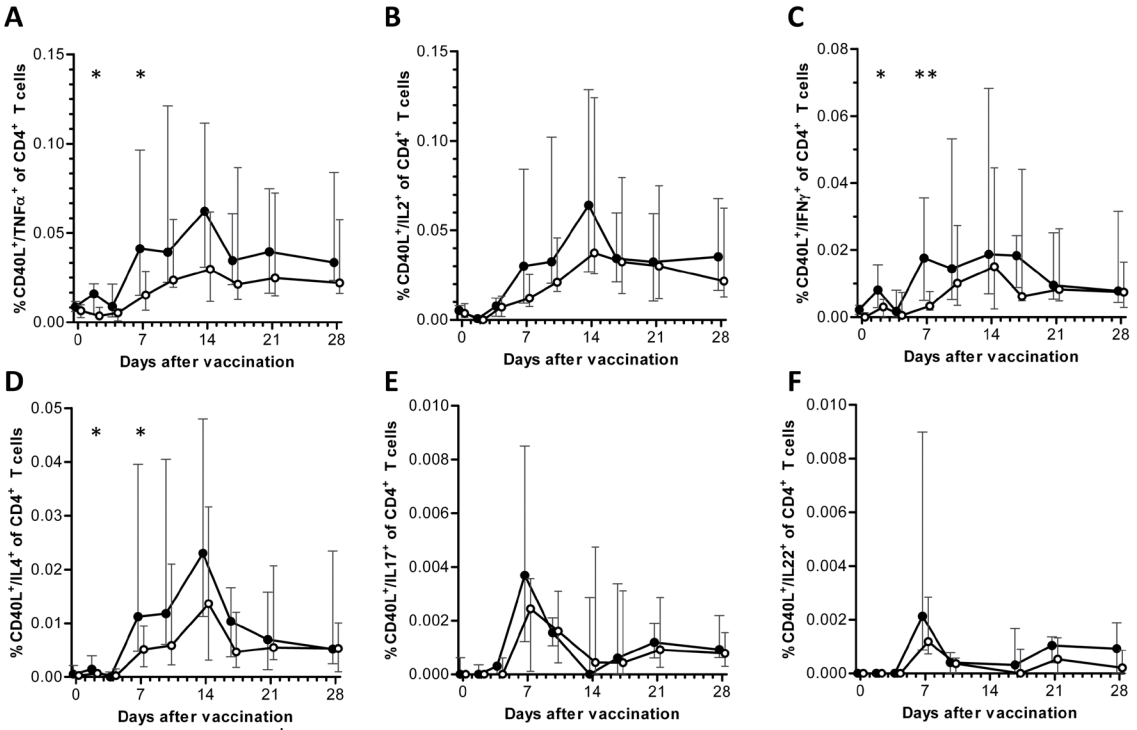
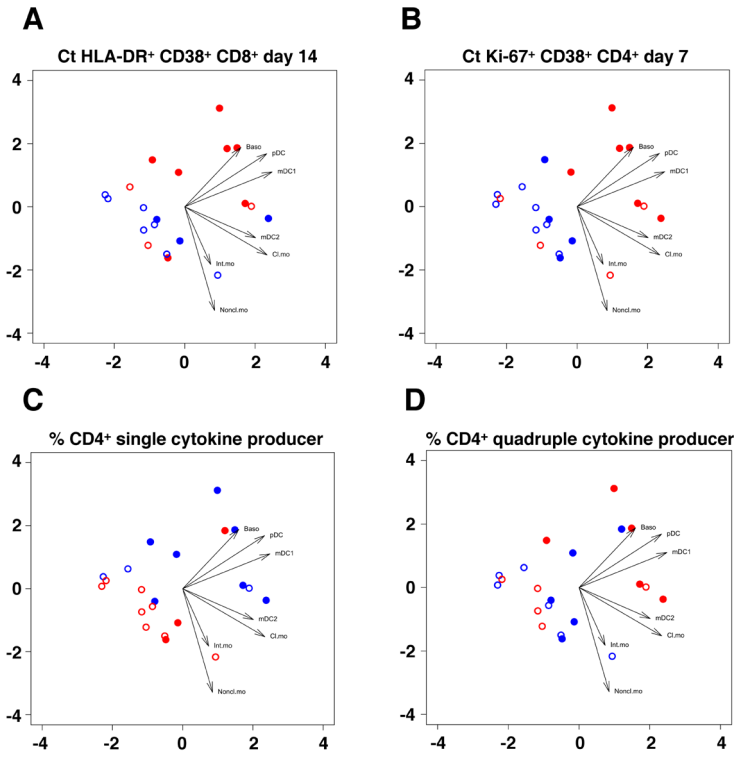
6. Appendix

6.1 Supplementary figures



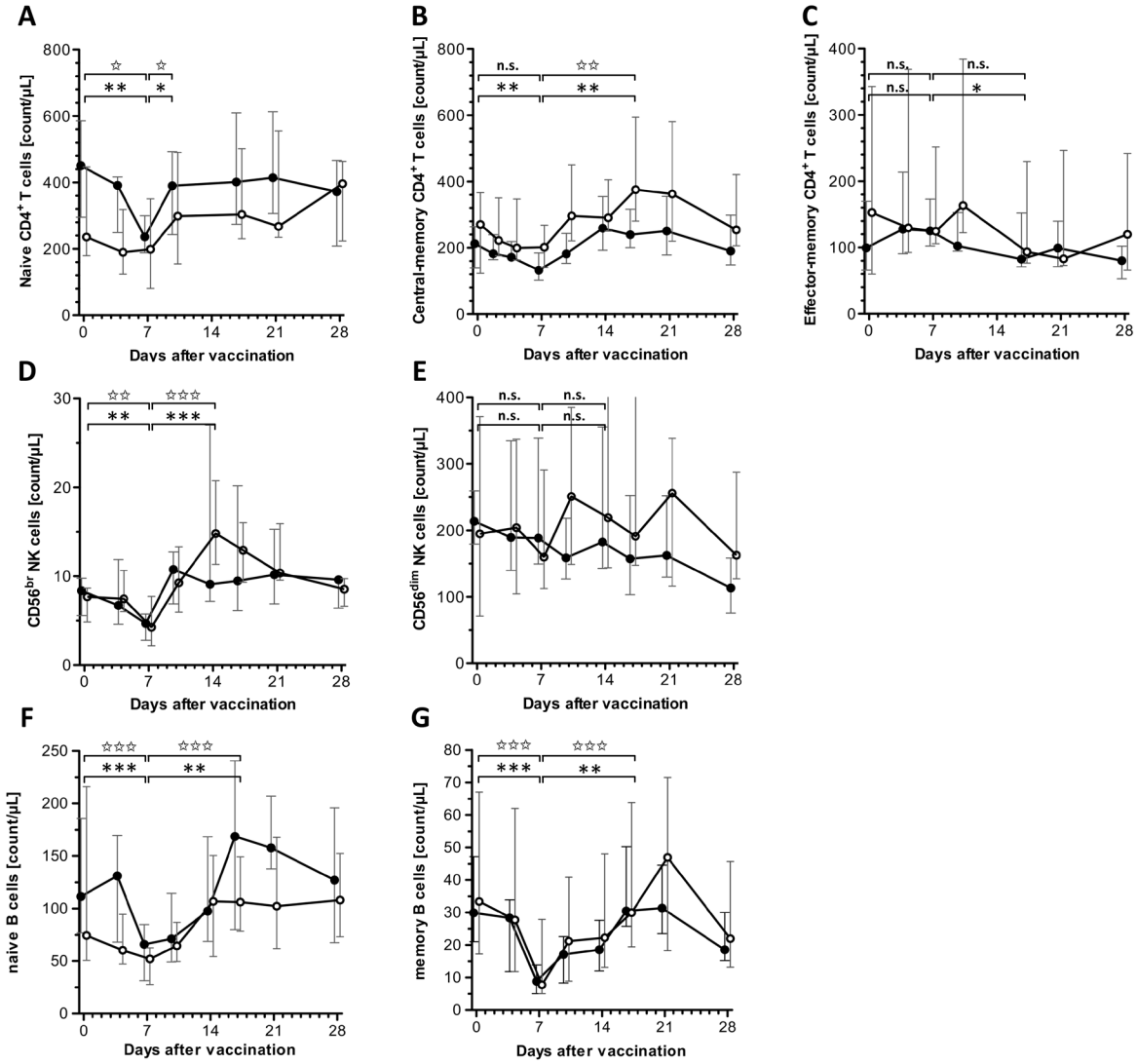
Suppl. fig. 1: YF-specific CD4$^+$ T-cell response after re-stimulation with YF-vaccine (frequencies)

(**A-F**) Frequencies of YF-specific CD4$^+$ T cells assessed by co-expression of CD40L and a cytokine (CD40L/TNFα: day 2 p=0.03; day 7 p=0.03; CD40L/IFNγ: day 2 p=0.05; day 7 p=0.006; CD40L/IL4: day 2 p=0.04; day 7 p=0.05). Lines indicate the median with interquartile error bars. Young: filled circles; elderly: open circles



Suppl. fig. 2: Overlay of selected YF immune signatures on innate PCA

Color overlay of median separated values of (**A**) numbers of activated HLA-DR$^+$/CD38$^+$ CD8$^+$ T cells at day 14, (**B**) numbers of Ki-67$^+$ CD38$^+$ CD4$^+$ T cells at day 7, (**C**) qualitative YF-specific single and (**D**) quadruple CD4$^+$ functionality on the PCA of innate immunity at day 4. Red: values >median; Blue: values <median; Young: filled circles; Elderly: open circles



Suppl. fig. 3: Temporospatial distribution and composition of immune cells in peripheral blood

(**A-G**) Absolute cell counts of (A) naive $CD4^+$, (B) CM $CD4^+$, (C) EM $CD4^+$, (D) $CD56^{br}$ NK, (E) $CD56^{dim}$ NK, (F) naive B and (G) memory B cells in peripheral blood in the course of the vaccine-induced immune response. Lines indicate the median with interquartile error bars. Wilcoxon paired signed rank tests were performed for each age group. Asterisks indicate significances in young and stars in elderly vaccinees. P-values are listed in suppl. table 3. Young: filled circles; elderly: open circles

6.2 Supplementary tables

Suppl. table 1: P-values of innate cell subsets

Subset	Day	P-Value
Class. monocytes ($CD14^{++}/CD16^{-}$)	7	0.02
Noncl. monocytes ($CD14^{+}/CD16^{+}$)	10	0.02
	21	0.01
	28	0.0005
Interm. monocytes ($CD14^{++}/CD16^{+}$)	10	0.03
Plasmacytoid DCs	0	0.004
	4	0.0006
	7	0.005
	10	0.03
	14	0.009
	17	0.006
	21	0.02
	28	0.03
$CD11c^{+}$ myeloid DCs	0	0.05
	4	0.03
Basophils	7	0.02
Neutrophils	0	0.03

Suppl. table 2: P-values of immune cell migration patterns I

Subset	Comparison	Age	P-Value
$CD19^{+}$ B cells	Day 0 → Day 7	Young	0.001
		Old	0.0002
	Day 7 → Day 17	Young	0.002
		Old	0.0002
$CD4^{+}$ T cells	Day 0 → Day 7	Young	0.001
		Old	0.006
	Day 7 → Day 14	Young	0.002
		Old	0.001
$CD8^{+}$ T cells	Day 0 → Day 7	Young	0.001
		Old	0.02
	Day 7 → Day 17	Young	0.03
		Old	0.0005
Naive $CD8^{+}$ T cells	Day 0 → Day 7	Young	0.001
		Old	0.0002
	Day 7 → Day 10	Young	0.003
		Old	0.0005
Central-memory $CD8^{+}$ T cells	Day 0 → Day 7	Young	0.001
		Old	0.0002
	Day 7 → Day 14	Young	0.003
		Old	0.002
Effector-memory $CD8^{+}$ T cells	Day 0 → Day 7	Young	0.76
		Old	0.94
	Day 7 → Day 17	Young	0.63
		Old	0.02
Effector $CD8^{+}$ T cells	Day 0 → Day 7	Young	0.24
		Old	0.19
	Day 7 → Day 17	Young	0.92
		Old	0.06

Suppl. table 3: P-values of immune cell migration patterns II

Subset	Comparison	Age	P-Value
Naive $CD4^+$ T cells	Day 0 → Day 7	Young	0.004
		Old	0.02
	Day 7 → Day 10	Young	0.03
		Old	0.03
Central-memory $CD4^+$ T cells	Day 0 → Day 7	Young	0.04
		Old	0.19
	Day 7 → Day 17	Young	0.008
		Old	0.004
Effector-memory $CD4^+$ T cells	Day 0 → Day 7	Young	0.07
		Old	0.82
	Day 7 → Day 17	Young	0.04
		Old	0.73
$CD56^{br}$ NK cells	Day 0 → Day 7	Young	0.003
		Old	0.002
	Day 7 → Day 14	Young	0.001
		Old	0.0002
$CD56^{dim}$ NK cells	Day 0 → Day 7	Young	1
		Old	0.62
	Day 7 → Day 14	Young	0.28
		Old	0.17
Naive B cells	Day 0 → Day 7	Young	0.001
		Old	0.001
	Day 7 → Day 17	Young	0.004
		Old	0.0005
Memory B cells	Day 0 → Day 7	Young	0.001
		Old	0.001
	Day 7 → Day 17	Young	0.004
		Old	0.0005

6.3 List of abbreviations

ACA	Automated clustering analysis
AID	Activation-induced cytidine deaminase
ASC	Antibody-secreting cell
BSA	Bovine serum albumin
BZLF1	BamHI Z Leftward reading Frame 1 (an EBV immediate early-protein)
CCR	CC chemokine receptor
CD	Cluster of differentiation
CM	Central-memory
CMV	Cytomegalovirus
CTL	Cytotoxic T lymphocyte
DAPI	4',6-Diamidino-2-Phenylindole
DC	Dendritic cell
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxide
EBNA1	Epstein-Barr virus nuclear antigen 1
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
Eff	Effector
EM	Effector-memory
FACS	Fluorescence-activated cell sorting
FoxP3	Forkhead box P3
GFV	Gelbfiebervirus
Grz	Granzyme
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
ICS	Intracellular cytokine staining
IE-1	Human cytomegalovirus immediate early-protein 1
IFN	Interferon
Ig	Immunoglobulin
IIFA	Indirect immunofluorescence assay
IL	Interleukin
LN	Lymph node
MAIT cells	Mucosal associated invariant T cells
mDCs	Myeloid dendritic cells
min	Minute
N	Naive
NCBI	National Center for Biotechnology Information
NK cells	Natural killer cells
NKT cells	Natural killer T cells
NS4b	Nonstructural protein 4b

PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCA	Principal component analysis
pDCs	Plasmacytoid dendritic cells
pp65	Human cytomegalovirus phosphoprotein 65
PRNT90	Plaque reduction neutralization test 90
RANTES	Regulated on activation, normal T cell expressed and secreted
RKI	Robert Koch-Institute
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
RT	Room temperature
RTE	Recent thymic emigrants
RT-PCR	Real-time polymerase chain reaction
SARS	Severe acute respiratory syndrome
SIV	Simian immunodeficiency virus
TBE	Tick-borne encephalitis
TCR	T-cell receptor
T_H1	Type 1 T Helper cells
T_H17	Type 17 T Helper cells
T_H2	Type 2 T Helper cells
TLRs	Toll-like receptors
TNFα	Tumor necrosis factor α
T_{reg}	Regulatory T cells
VZV	Varicella zoster virus
WHO	World Health Organization
YEL-AND	YFV-associated neurotropic disease
YEL-AVD	YFV-associated viscerotropic disease
YF	Yellow fever
YF-SAE	YFV-related severe adverse events
YFV	Yellow fever virus

6.4 List of figures

6.5 List of tables

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Publications

Wilhelm TR, Taddeo A, Winter O, Schulz AR, Mälzer JN, Domingo C, Biesen R, Alexander T, Thiel A, Radbruch A, Hiepe F, Gerl V.
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Jürchott K, Schulz AR, Meier S, Bozzetti C, Rachwalik D, Mälzer JN, Stervbo U, Babel N, Thiel A, Neumann A.
Highly predictive model of response to H1N1 Influenza strain after seasonal Influenza vaccine.
(In revision at PLOS One)

Presentations at conferences and meetings

Low thymic activity and dendritic cell numbers determine the human immune response to primary Yellow Fever-17D vaccination in the elderly (Poster)
25th Annual Meeting of the German Society for Cytometry, 2015, Berlin, Germany

Low thymic activity and dendritic cell numbers determine the human immune response to primary Yellow Fever-17D vaccination in the elderly (Poster)
ECI Satellite Symposium: Immunosenescence - Hot Topics & Interventions, 2015, Vienna, Austria

Compromised Innate and Adaptive Immune Responses during Yellow Fever Vaccination in Elderly - a Multiparametric Longitudinal FACS Study (Oral presentation)
CYTO 2013, XXVII Congress of the International Society for the Advancement of Cytometry (ISAC), San Diego, USA

Multiparametric High-Throughput Characterization of the Immune Response after Seasonal Influenza Vaccination (2011/12) in Young and Elderly Adults (Poster)
CYTO 2012, XXVII Congress of the ISAC, Leipzig, Germany

Multiparametric Characterization of the Immune Response after Yellow Fever 17D-Vaccination in Young and Elderly Adults (Poster)
Joint Annual Meeting of the Italian Society for Immunology, Clinical Immunology and Allergology and the German Society for Immunology 2011, Riccione, Italy

Phenotyping the Yellow Fever Virus-17D specific $CD8^+$ T-Cell Response (Oral presentation)
14th German Meeting on T-cells: Subsets and functions, 2011, Marburg, Germany

Selbstständigkeitserklärung

Ich erkläre hiermit, dass ich die vorliegende Arbeit selbständig und nur unter Verwendung der angegebenen Hilfen und Hilfsmittel nach §7, Absatz 3 der Promotionsordnung vom 20. April 2011 der Mathematisch-Naturwissenschaftlichen Fakultät I der Humboldt Universität zu Berlin angefertigt habe. Wurden Ergebnisse in Kooperation produziert, ist dies entsprechend angegeben.

Berlin, 01.12.2015

Axel Schulz